UNEXPLOITED GERMPLASM, NATURAL MUTATIONS, AND SELECTED IN VITRO TECHNIQUES FOR CITRUS CULTIVAR IMPROVEMENT

By

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KEY TO ABBREVIATIONS

BA = N-(phenylmethyl)-1 H-purin-6-amine; benzyladenine.

CP1 = Callus proliferation medium.

EME = Medium for growth of embryogenic callus.

GM1 = Medium for germination of seeds.

GTF = Grandis-type forbidden fruit.

Gy = Gray (a measure of radiation; 10 Gy = 1 krad).

i.d. = Inside diameter.

MT = Murashige and Tucker *Citrus* medium (Murashige and Tucker, 1969).

NAA = 1-naphthaleneacetic acid.

RM1 = Root induction medium.

SCE = Sister chromatid exchange.

SIM = Shoot induction medium.

SPM2 = Fafard No. 2 (Conrad Fafard, Springfield, MA) soilless potting mix.

SF = Shaddette-type forbidden fruit.

2,4-D = (2,4-dichlorophenoxy)acetic acid.

2,4,5-T = 2-(2,4,5-Trichlorophenoxy)propionic acid.

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Breeding of *Citrus* by sexual hybridization has not been an effective strategy for cultivar improvement because of long juvenile stages and widespread apomixis. Investigations of unexploited genetic resources or systems of evaluation may identify methods to increase the rate of genetic advance. One potential germplasm resource, two sources of genetic mutants, and one novel method of in vitro evaluation for disease resistance were examined during this study. The potential value of each project was critically reviewed.

A diverse population of *Citrus*, known as forbidden fruit, was discovered in Saint Lucia. Historical records, morphological characteristics, and isozyme evidence indicated that this population was closely related to grapefruit. High degrees of zygotic embryony and diversity within the population may make it a useful source of breeding parents.

Significant morphological variation was observed during initial growth among *Citrus* plants regenerated from callus cultures by organogenesis. Many of the aberrant plants were indistinguishable from controls after a second cycle of vegetative regrowth. The frequency of genetic mutation that could be measured as stable morphological change in young plants was too low to allow efficient mutant recovery.

Fruit sector chimeras were observed among eight *Citrus* cultivars at a frequency of 0.01 to 0.27 percent in commercial packinghouses. Mutants were recovered from some sectored fruit as autotetraploid seedlings. Several kinds of sectors with potential value as sources of genetic mutations were described.

In vitro response of etiolated seedling shoots to *Phytophthora parasitica* was measured as stem discoloration. The non-host, or hypersensitive, response observed in *Poncirus trifoliata* and *Severinia buxifolia* was clearly distinguished from the susceptible reaction of eight *Citrus* selections and hybrids. However, two *Citrus* × *Poncirus* hybrids with useful levels of field resistance to this pathogen exhibited a highly susceptible reaction in vitro. This technique may prove useful in the study of resistance mechanisms, but probably will not be effective for population screening.

CHAPTER 1
INTRODUCTION

The genus *Citrus* supplies several fruit crops of great importance in tropical and subtropical regions of the world. The species of greatest economic value as scions are *Citrus sinensis* (L.) Osbeck (sweet oranges), *C. reticulata* Blanco (mandarins), *C. ×paradisi* Macfadyen (grapefruits), *C. limon* (L.) Burm.f. (lemons), *C. aurantifolia* (Christm.) Swingle (limes), *C. grandis* (L.) Osbeck (pummelos), and a number of interspecific hybrids, especially tangors (*C. reticulata* × *C. sinensis*) and tangelos (*C. ×paradisi* × *C. reticulata*). In addition, many other species (e.g. the sour orange, *C. aurantium* L.), close relatives (e.g. *Poncirus trifoliata* (L.) Raf. and *Fortunella margarita* (Lour.) Swingle), and hybrids (e.g. 'Carrizo' citrange, *P. trifoliata* × *C. sinensis*) are important as rootstocks or specialty crops (Hodgson, 1967; Morton, 1987; Swingle and Reece, 1967). This dissertation is focused on the genetic improvements desired for the world's citrus industries, including simplified cultural demands, improved fruit quality, increased yield, and reduced susceptibility to cold, diseases and pests. Achieving significant genetic improvement is hampered by several major obstacles: a long juvenile phase, widespread nucellar polyembryony, the heterozygosity of most clones, and the lack of screens for selection of favorable young plants.

Citrus spp. are woody perennials with an extended juvenile period during which no flowering occurs. *Citrus* species vary considerably in the length of the juvenile phase. Limes generally require only 2-3 years from seeding to first fruiting, while sweet oranges may take 10 or more years before the initiation of flowering (Furr et al., 1947; Sherman and Lyrene, 1983). The long life cycle delays evaluation of hybrid fruiting characteristics and is a major factor limiting breeding progress. In addition, most commercially important *Citrus* cultivars and species are highly polyembryonic (Frost and Soost, 1968). These selections produce seeds composed of multiple nucellar (apomictic) embryos. Nucellar embryos are generally more well developed and vigorous than the single zygotic embryo that may or may not survive in the polyembryonic clones. As a result, most seedlings from polyembryonic species are genetically identical to the maternal parent even when harvested from fruit produced by controlled pollination. This factor severely limits sexual hybridization within all *Citrus* except those species with monoembryonic selections (*C. medica* L. [citrons], *C. grandis*, and part of *C. reticulata*). High heterozygosity has been noted in most species of *Citrus* (Cameron and Frost, 1968; Frost, 1926; Hagedoorn and Hagedoorn, 1914) and, when combined with nucellar embryony and extensive vegetative propagation of desirable genotypes, has resulted in the accumulation of many deleterious recessive alleles in existing germplasm collections (Cameron and Frost, 1968). Hybridization among these heterozygous types frequently results in weak (Cameron and Frost, 1968; Frost, 1943; Torres, 1936) and/or highly variable progeny (Cameron and Frost, 1968; Swingle, 1913).

The limitations placed on sexual hybridization by nucellar embryony, the unpredictability of the results of successful hybridization, and the long juvenile phase are problems that are exacerbated by the lack of methods for evaluation of important characteristics in non-mature seedlings (Soost and Cameron, 1975). Frequently, it is difficult to identify the few true zygotic seedlings that may be obtained from a large population of predominantly apomictic seedlings without waiting for fruiting or using expensive and time-consuming biochemical techniques (Bade et al., in press; Scora and Kumamoto, 1983; Soost et al., 1980; Torres et al., 1978, 1985).

With this perspective, *Citrus* breeders have directed considerable attention toward alternatives to sexual hybridization for genetic improvement. The most notable successes have been in the fields of somatic hybridization (Grosser and Gmitter, 1989, 1990; Grosser et al., 1989; Kobayashi and Ohgawara, 1988; Ohgawara et al., 1989; Vardi et al., 1987, 1989) and somatic mutagenesis (Hearn, 1984, 1986; Hensz, 1971, 1977, 1985; Russo et al., 1981; Spiegel-Roy and Kochba, 1973a; Spiegel-Roy et al., 1985; Starrantino et al., 1988a, 1988b). More recent interest has been directed toward the application of genetic engineering to *Citrus* genetic improvement. Although *Citrus* molecular manipulation is still in its infancy, the success of similar genetic engineering in other plant species (Deshayes et al., 1985; Gasser and Fraley, 1989; Horsch et al., 1985; Prols et al., 1989; Sanford, 1988; Saunders et al., 1989; Tomes et al., 1990; Weising et al., 1988) has encouraged considerable optimism. Characteristics such as virus (Nelson et al., 1987; Powell Abel et al., 1986), insect (Fischhoff et al., 1987; Vaeck et al., 1987), and herbicide

(Comai et al., 1985; Shah et al., 1986) resistance, as well as others (Hiatt et al., 1989; Murai et al., 1983; Ow et al., 1986; Smith et al., 1990; Weising et al., 1988) have been engineered successfully into plants. The rapid advance in understanding of codon usage (Campbell and Gowri, 1990) and regulatory sequences (Benfey and Chua, 1989), along with the cloning of unique genes or determination of complete amino acid sequences for other proteins influencing cold tolerance (Cutler et al., 1989; Lee, 1989), sweetness (Inglett and May, 1968; Iyengar et al., 1979; Kennedy et al., 1988; Ledeboer et al., 1984), insect resistance (Fishman et al., 1984; Lazarovici et al., 1984; Quicke, 1988), bacterial resistance (Casteels et al., 1989), and other characteristics (Gasser and Fraley, 1989; Weising et al., 1988) may indicate the much broader applicability of these techniques. There are already some examples of successful production of genetically transformed woody species (James et al., 1989; McGranahan et al., 1988).

Unfortunately, each of these promising techniques appears to have significant constraints. Somatic hybridization offers many opportunities for useful genetic combinations, but will probably be limited by the inability to access desirable traits controlled by recessive genes or to eliminate dominant negative traits. In vivo somatic mutation of *Citrus* has proven effective in the induction of three specific types of useful genetic changes (i.e. increased pigmentation [Hensz, 1971, 1977, 1985], decreased acidity [Yen, 1987], and decreased seediness [Hearn, 1984, 1986]) and will probably remain confined in scope. Plant genetic engineering presents a number of ecological problems and social complications (Doebley, 1990; Ellstrand and Hoffman, 1990; Hoffman, 1990;

Pimentel et al., 1989; Raffa, 1989; Wilson, 1990) that may interfere with successful deployment of modified plants. In addition, specific molecular modification of plant genomes will most likely be restricted to characteristics under simple genetic control for some time to come, and many of the limits confining conventional breeding (e.g. greater energy spent on resistance to environmental factors = less energy to put into yield) will also restrain genetic engineering. With the exception of induced mutagenesis of mature budwood (in vivo mutagenesis) that avoids the juvenile phase, none of these methods addresses the often more significant problem of evaluating the "potentially" improved genotypes that they produce. Somatic hybridization and genetic transformation utilize plant regeneration from single cells and therefore revert to the juvenile stage.

Considerable value may be derived from the development of additional approaches to genetic improvement of *Citrus*, both in terms of inducing genetic variation and evaluating germplasm. This dissertation is devoted to the investigation of four such areas: the Caribbean forbidden fruit, somaclonal variation/in vitro mutagenesis, fruit sector chimeras, and in vitro screening for resistance to *Phytophthora*. The Caribbean forbidden fruit is a recently identified, genetically diverse population of grapefruit-like *Citrus* that should provide valuable breeding parents with good fruit quality and a high degree of zygotic embryony. Induction of variation in tissue cultures (by somaclonal variation or applied mutagenesis) may provide alternative methods for inducing favorable somatic mutations in existing cultivars. Fruit sector chimeras are another source of somatic (as opposed to germ line) mutations that may

allow effective selection of desirable mutations at the outset of experimentation, thus minimizing the high frequency of deleterious mutations commonly associated with induced mutagenesis. Finally, evaluation of clone resistance to *Phytophthora* in vitro could supply important information about resistance mechanisms. If correlated with field response, such a test could significantly expedite germplasm evaluation, especially in conjunction with in vitro methods of inducing genetic variation.

CHAPTER 2

REVIEW OF THE LITERATURE

Introduction

As a background for the dissertation research described herein, a thoughtful review of the pertinent literature has been conducted in each of the subject areas. Appropriate contemporary and historical literature was examined to provide as much relevant information as possible on the Caribbean forbidden fruit/grapefruit, somaclonal variation, gamma irradiation of tissue cultures, fruit sector chimeras, and in vitro evaluation of *Phytophthora* resistance. Following are concise, but thorough and well documented summaries of these topics.

Forbidden Fruit

Citrus ×paradisi (grapefruit) is the only widely recognized *Citrus* species that is considered to have arisen in the western hemisphere. Thorough searches of the *Citrus* regions in Asia and the Mediterranean have revealed no grapefruit, except those introduced from the new world (Hodgson, 1967; Kumamoto et al., 1987; Scora et al., 1982; Swingle and Reece, 1967). Grapefruit is now considered to be an apomictically reproducing hybrid species that originated in the West Indies during the 17th or 18th century. On the basis of historical, morphological, and biochemical considerations, the most probable parents of grapefruit are

C. sinensis and *C. grandis* (Chapot, 1950; Scora and Kumamoto, 1983; Scora et al., 1982). *C. grandis* (syn. *C. maxima* [Burm.] Merrell. and *C. decumana* L.) and *C. sinensis* were present in the West Indies by 1692 (Scora et al., 1982; Sloane, 1696). Grapefruit probably originated between this time and the 19th century by one or a series of natural hybridizations between the two species. However, a more complex scenario involving multiple hybridization events and other species is possible within the present state of knowledge.

The grapefruits, as they are known in the United States citrus industry, are a group of morphologically similar and very closely related selections (Cooper, 1982; Hodgson, 1967; Ziegler and Wolfe, 1961). The most common cultivars in Florida are 'Marsh' and 'Ruby Red' (Florida Dept. Agr., 1988a, 1988b), two commercially seedless cultivars that differ principally in flesh and rind coloration (Hodgson, 1967). 'Ruby Red' was first identified in 1929 as a limb sport on one tree of 'Thompson' pink grapefruit by A.E. Henninger in Texas (Hodgson, 1967; Ziegler and Wolfe, 1961). 'Thompson' was itself selected as a limb sport of 'Marsh' in 1913 (Hodgson, 1967; Ziegler and Wolfe, 1961). Many other red-fleshed limb sports of 'Marsh' or 'Thompson' have been identified, but most were considered to be essentially identical with 'Ruby Red' (Waibel, 1953; Ziegler and Wolfe, 1961). Another cultivar that has achieved some popularity/notoriety is 'Star Ruby', a darker-red fleshed cultivar that originated from an irradiated seedling (probably mutated nucellar, not zygotic) of the cultivar 'Hudson' (Hensz, 1971, 1977). 'Hudson' was a limb sport of the cultivar 'Foster', that was in turn a limb sport of the cultivar 'Walters' (Hodgson, 1967). Three new darker red-fleshed

cultivars ('Ray', 'Henderson' [or a nucellar seedling of 'Henderson' known as 'Flame' in Florida], and 'Rio Red') that are attracting tremendous attention in Florida, Texas, and California (Anonymous, 1988, 1989a, 1989b; Fairchild and Teague, 1987; Nauer et al., 1988) were produced as natural or irradiation-induced bud sports from 'Ruby Red' (Hensz, 1978, 1981, 1985). The remaining commercial grapefruit cultivar is 'Duncan', a seedy, highly flavored selection that is now declining in significance and probably originated from a seedling planted in Florida about 1830. 'Duncan' was first introduced and propagated around 1892 (Hodgson, 1967; Ziegler and Wolfe, 1961). A grove of grapefruit at Safety Harbor, Florida, that was planted about 1823 by Count Don Phillippe either directly produced 'Duncan' (Robinson, 1947; Ziegler and Wolfe, 1961), or was the source of the seed that produced this cultivar (Hodgson, 1967). This same grove of grapefruit probably also was the source, directly or indirectly, for the cultivars 'Walters' and 'Marsh' (Cooper, 1982; Ziegler and Wolfe, 1961).

Count Phillippe's grapefruit grove was planted by importation of seed or trees from the Bahamas (Robinson, 1952; Anonymous, 1921; Ziegler and Wolfe, 1961), Cuba (Webber, 1943), or somewhere else in the West Indies. There are no other reports of grapefruit germplasm importation into Florida, although Hume (1926) suggested that it may have been brought to the state by the Spaniards between 1513 and 1821.

Other *Citrus* cultivars similar to grapefruit have been named (including 'Oroblanco' [Soost and Cameron, 1980a, 1980b], 'Melogold' [Soost and Cameron, 1985, 1986], 'Triumph', etc. [Hodgson, 1967]). Many of these are thought or known to be the result of hybridization between

one of the aforementioned grapefruit cultivars and *C. sinensis*, *C. reticulata*, or *C. grandis*. None of these grapefruit hybrids has been enough like grapefruit to compete with 'Marsh', 'Ruby Red', or the other red-fleshed sports for commercial importance (Hodgson, 1967).

Despite the uniqueness and commercial success of the popular grapefruit cultivars, they are not without their faults, including irregular fruit size, cold sensitivity, disease susceptibility, and excessive fruit bitterness. Richard Hensz pointed out that

> The improvements attributed to all of the new grapefruit cultivars that have developed since the beginning of commercial production have been towards enhancement for consumer acceptance with no real improvement in other horticultural characteristics of the tree or fruit. Presently grown seedless cultivars are no more vigorous, disease resistant, productive, nor have better eating quality than the white seedy types grown in the early days of the grapefruit industry. In fact, eliminating the seeds has resulted in the production of fruit of comparably smaller size and frequently lower yields.
> (Hensz, 1977; p. 582)

The 'Star Ruby' cultivar (developed by R.A. Hensz) has been observed to be less resistant than less darkly pigmented cultivars to many environmental stresses, including *Phytophthora* foot rot, herbicides, and sunburning (Hensz, 1977). Genetic improvement of grapefruit has been frustrated by the long juvenile period (Frost, 1943; Furr et al., 1947), high degree of nucellar embryony (Frost and Soost, 1968), and very limited variability among the known selections (Hodgson, 1967). Attempts to overcome these obstacles with existing selections have not been successful (Furr and Reece, 1946; Snowball et al., 1988; Soost and Cameron, 1975; Wantanabe et al., 1970).

Genetic improvement of grapefruit would benefit by the identification of grapefruit selections with a shortened juvenile period, and greater zygotic embryony and genetic diversity. The loss of genetic diversity that results when population size is reduced to a small number of individuals has been recognized (Carson, 1970, 1971; Mayr, 1963; Nei et al., 1975; Prakash, 1972; Wright, 1931). Such a genetic "bottleneck" may have occurred in the formulation of current populations of North American grapefruit from the single Phillippe introduction (Bowman and Gmitter, 1990a). Many of the characters desired for grapefruit genetic improvement might be obtained from a diverse wild population, but no such wild grapefruit have been identified or examined as sources of additional germplasm. Maximum genetic diversity for crop species has frequently been associated with the center of species origin (Clement, 1989; Harlan, 1971; Hawkes, 1983; Vavilov, 1951). It is uncertain whether this trend would apply to species (like grapefruit) that originated as interspecific hybrids in the relatively recent past (200-300 years). Regardless, centers of plant diversity not associated with place of origin have been documented (Harlan, 1975; Peeters, 1988), and any existing wild populations of grapefruit in the West Indies would seem to be potential sources of unique alleles and genetic combinations.

The origin of grapefruit has been discussed by several authors on the basis of the historical literature (Bowman and Gmitter, 1990a, 1990b; Kumamoto et al., 1987; Robinson, 1952; Scora et al., 1982). The species name now given to grapefruit, *Citrus paradisi*, was assigned in 1830 to a kind of *Citrus* from Jamaica identified with the common name of "Forbidden Fruit" (Macfadyen 1830, 1837). At that time, Macfadyen indicated that

there were two botanical varieties of this species in Jamaica, one known as "Forbidden Fruit" and the other known as "Barbadoes Grape Fruit" (p. 304). Subsequent descriptions of *Citrus* in West Indian literature have typically used the names grapefruit and forbidden fruit as synonyms (Fawcett and Rendle, 1920; Freeman and Williams, 1927; Stehle et al., 1937; Stout, 1982). The most recent report of forbidden fruit as a distinct selection was from Bermuda (Britton, 1918), where it was described as a particular small fruited selection of *C. grandis*.

About the same time as Macfadyen's application of the specific epithet *C. paradisi*, a resident of Jamaica, Maycock (1830), stated "My notes of the Grape-fruit and Forbidden-Fruit Trees, I am sorry to find, are too imperfect to enable me to say with certainty that they are specifically distinct, although I am inclined to think they are. I feel quite certain they are not varieties of *C. decumana*" (p. 318). Tussac (1824) also made a possible connection between the two selections when he described the Jamaican forbidden fruit as "fruit defendu, ou smaller schaddoc, petit chadec" (p. 74) with fruit borne in clusters like grapes. Prior to 1830, the only other use of the name grapefruit in the literature was by John Lunan, who described a variety of Jamaican shaddock "known by the name of grape-fruit on account of its resemblance in flavour to the grape" (Lunan, 1814; p. 171). These early descriptions, therefore, suggest two possible origins for the name "grapefruit," the flavor and/or the fruit clustering.

Jamaican forbidden fruit was described by several authors before Macfadyen (Browne, 1756, 1789; Lunan, 1814; Tussac, 1824), although they did not always agree on morphological characteristics. The first

description of forbidden fruit was from Barbados, an island some 1800 km southeast of Jamaica (Hughes, 1750):

FORBIDDEN-FRUIT-TREE

> The trunk, leaves, and flowers of this Tree, very much refemble thofe of the Orange-tree.
>
> The Fruit, when ripe, is fomething longer and larger than the largeft Orange; and exceeds, in the Delicacy of its Tafte, the Fruit of every Tree in this or any of our neighbouring Iflands.
>
> It hath fomewhat the Tafte of a Shaddock; but far exceeds that, as well as the beft Orange, in its delicious Tafte and Flavour.
>
> This is delineated in Plate VII. (p. 127)

Fourteen other kinds of *Citrus* found growing in Barbados at that time were described by Hughes, and one of these, the Guiney orange, he suggested should be called the "sour forbidden fruit." Hughes also produced a drawing of the forbidden fruit tree that showed pyriform fruit, no spines, and leaves without distinctively alate petioles. Hughes' depiction of the Barbados forbidden fruit tree without spines or winged petioles is at odds with the description of spines and alate petioles given by Browne (1756) and Macfadyen (1830, 1837) for the forbidden fruit of Jamaica. In addition, Macfadyen chose the pyriform fruit shape as a characteristic that distinguished the "Barbadoes Grape Fruit" from the "maliformis" Forbidden Fruit. Maycock (1830) indicated that Hughes had no botanical expertise, and we are left with uncertainty as to whether Hughes, in Barbados, was describing the same selection known as forbidden fruit by Macfadyen in Jamaica. Kumamoto et al. (1987) indicated that they believed the golden orange described by Hughes was actually what later became known as grapefruit, and that "observers tended to confuse the grapefruit with the forbidden fruit, believing them to be the same fruit

or varieties of the same species" (p. 100). Although some confusion may have occurred among these early authors, there seems little reason to question the close relationship between forbidden fruit and grapefruit agreed upon by Macfadyen (1830, 1837) and Maycock (1830) without examination of botanical specimens. Unfortunately, good herbarium specimens (personal communication, R.A. Howard, Harvard) or living selections of Jamaican and Barbados forbidden fruits do not exist. Kumamoto et al. (1987) believed "the forbidden fruit tree became extinct, not having been mentioned after the 19th century, [while] the grapefruit flourished and spread throughout the West Indies" (p. 100). Both Hughes (1750), by his forbidden fruit and sour forbidden fruit, and Macfadyen (1830), by his two botanical varieties of forbidden fruit, indicated that there was considerable variability within the "forbidden fruit." This variation may simply be the result of normal intraspecific genetic diversity, not confusion. Controversy over what constitutes a taxonomic group within the genus *Citrus* has been common (Barrett and Rhodes, 1976; DeCandole, 1824; Green et al., 1986; Hodgson, 1961, Hodgson et al., 1963a, 1963b, 1963c; Scora, 1988; Scora and Kumamoto, 1983; Swingle, 1914, 1943; T. Tanaka, 1927a, 1935, 1954, 1961, 1977; Y. Tanaka, 1948) and is obviously fueled by the nature of apomixis and the consequent lack of traditional species groups in agamic complexes (Crow and Kimura, 1965; Stebbins, 1950). However, the accepted interspecific origin (*C. grandis* × *C. sinensis*) and monoembryonic ancestry (from *C. grandis*) of grapefruit indicates that some genetic differences were probable among seedlings from identical parentage. This natural genetic diversity may be sufficient to

explain the variation in forbidden fruit/grapefruit characteristics among descriptions by early authors.

Although the grapefruit is generally believed to have originated in the Caribbean, there are some indications that forbidden fruit (and possibly grapefruit) may have been (or were derived from) one of the selections of *Citrus* previously known in the Mediterranean region as "apple of Adam" or "Adam's apple." Bonavia (1888) produced figures of a kind of *Citrus* fruit being sold in England as "forbidden fruit." He noted that this was not the same as the pummelo, and indicated that one specimen had very smooth skin, a solid center, and was "sub-acid and sweet, and slightly bitter" (Bonavia, 1888; plate XCII), while a second was smaller and pyriform. Bonavia (1888) also wrote (in reference to the forbidden fruit) "Gallesio [1811] says the Crusaders found the *Pomo d'Adamo* in Palestine, and that it is not the Pompelmoess, the latter being a new citrus introduced from the East Indies" (plate XCII). Many selections of *Citrus* known as "apple of paradise" (Ferrari, 1646; Tolkowsky, 1938) or "apple of Adam" (Ferrari, 1646; Risso and Poiteau, 1872; Tolkowsky, 1938; Volkamer, 1708-1714) were described in the early citriculture literature from the Mediterranean, and one or more were probably introduced into the West Indies (Risso and Poiteau, 1872). Tolkowsky (1938) cites a Christian pilgrim who described about 1187 the "Adam's apple" growing in Palestine as "trees which bear fruit called Adam's apple (= the shaddock) wheron the marks of Adam's teeth may be right plainly seen" (p. 139). Tolkowsky also indicated that in the Middle Ages the citron and the shaddock were frequently known as "Adam's apple" or "apple of paradise," because of a Jewish tradition that considered the citron to

be the forbidden fruit described in the religious accounts of the Garden of Eden (Tolkowsky, 1938).

Somaclonal Variation

In the past several decades, there has been interest in applying plant tissue culture systems to propagation and genetic improvement of many crops. Most propagation techniques have made use of axillary or other preexisting meristems for proliferation of shoots and subsequent rooting. The propagation of clones in culture by way of preexisting meristems (mericlones) frequently has not resulted in significantly more variation than that observed with other methods of vegetative propagation (Conger, 1981). However, as more sophisticated techniques of plant culture, manipulation, and regeneration have been developed, it has been observed that some systems of culture may result in regenerates that are different in one or more significant characters from the original source. Larkin and Scowcroft (1981) have applied the term "somaclonal variation" to all such variability generated by plant cell culture and have suggested that this variation is of tremendous potential for plant improvement. Somaclonal variation has been the subject of many investigations and much speculation over the past decade (Brown and Lorz, 1986; D'Amato, 1985; DeWald and Moore, 1987; Evans, 1989; Evans et al., 1984; Evans and Sharp, 1986a, 1986b; Lee and Phillips, 1988; Orton, 1984; Reisch, 1983; Wersuhn, 1989). Evans et al. (1989) have been granted a United States patent for some methods of generating somaclonal variation.

In evaluation of variation among plants regenerated from tissue culture, it is important to distinguish between transient (epigenetic or

physiological) alterations in plant morphology and other more permanent (epigenetic or genetic) changes in the regenerated plants. However, the distinction between these two categories blurs somewhat in the cases of variation in methylation patterns (see below). Temporary changes of morphology and other characteristics have been commonly observed among plants regenerated from culture. Tissue culture propagation of strawberries produced temporary changes in many morphological characters (Marcotrigiano et al., 1984; Sansavini and Gherardi, 1980; Swartz et al., 1981). Shoemaker et al. (1985) observed an increase in susceptibility to root-rotting fungi among tissue culture-propagated strawberries. However, these plants reverted slowly to the normal resistant phenotype with increasing time out of culture, and the authors detected no evidence for genetic changes. The temporary changes in plants regenerated from tissue culture may rapidly revert to normal (Detrez et al., 1989; Griesbach, 1989), or only after extended growth and/or cycles of vegetative propagation (Lourens and Martin, 1987). These temporary changes may be the result of stress or a physiological adaption to the culture conditions, such as the habituation of tobacco pith tissues (Binns and Meins, 1973). Generally, the temporary alterations in phenotype are not of significance for propagation or cultivar improvement, except when they impair the ability to discriminate true genetic mutants. Most published reports of somaclonal variation have attempted to eliminate these transient effects and focus on changes that appear to be genetic or at least relatively long term.

Sugarcane was one of the first crops to obtain significant attention because of the clonal variation observed among regenerated plants.

Selections were recovered from tissue culture with increased yield and sucrose production (Heinz and Mee, 1971), as well as resistance to eyespot disease (Heinz et al., 1977), Fiji disease, and downy mildew (Krishnamurthi and Tlaskal, 1974). Subsequent selection of somaclones from cultures of major commercial varieties has been reported to yield variants with favorable changes in some morphological or horticultural characteristic (Heinz et al., 1977; Krishnamurthi, 1977; Larkin and Scowcroft, 1983; Liu and Chen, 1978; Maretzki, 1987). Many of these sugarcane clones have been tested over several years with no apparent loss of the acquired character (Orton, 1984), although other characters have exhibited some phenotypic instability (Maretzki, 1987).

Similar success with somaclonal variation was also reported for potato, which is a polyploid, vegetatively propagated crop like sugarcane. Shepard et al. (1980) were able to regenerate plants with changes in tuber skin color, tuber uniformity, maturity date, and numerous other characters from leaf protoplasts of a popular potato variety, 'Russet Burbank'. Some of these variants were reported to be horticultural improvements over the original clone. Plants also were obtained with increased resistance to early blight and late blight (*Phytophthora infestans*). Vegetative propagation of these variants over several generations did not degrade the acquired characteristics.

Type of change - morphology. Changes in many types of morphological or horticultural characteristics have been reported in somaclones, including leaf shape (Burk and Matzinger, 1976; Freytag et al., 1989; Osifo et al., 1989), foliage color (Osifo et al., 1989; Taliaferro et al., 1989), fertility (Daub and Jenns, 1989; Griesbach 1989; McPheeters and

Skirvin, 1989; Orton, 1984; Taliaferro et al., 1989), plant size (Eapen et al., 1989; Griesbach 1989; Jain et al., 1989; Taliaferro et al., 1989), growth habit (Freytag et al., 1989), seed color (George and Rao, 1983), time of flowering (Burk and Matzinger, 1976; Eapen et al., 1989; Ozias-Akins, 1989), fruit number per plant (Eapen et al., 1989), cold resistance (Galiba and Sutka, 1989; Lazar et al., 1988), insect resistance (Miles et al., 1981), disease resistance (Behnke, 1979, 1980; Daub and Jenns, 1989; Gengenbach et al., 1977; Hartman et al., 1984; Heath-Pagliuso et al., 1988; Heinz et al., 1977; Latunde-Dada and Lucas, 1983; Orton, 1984; Sacristan, 1982; Shahin and Spivey, 1986; Thanutong et al., 1983; Toyoda et al., 1989), resistance to other factors (Grandbastien et al., 1989; Kuehnle and Earle, 1989; McHughen, 1987; McHughen and Swartz, 1984; Ranch et al., 1983; Schaeffer et al., 1989; Shahin and Spivey, 1986; Wakasa and Widholm, 1987), alkaloid content (Burk and Matzinger, 1976), essential oil composition (Mathur et al., 1988), nutritive quality (Schaeffer and Sharpe, 1987; Schaeffer et al., 1989), and yield (Burk and Matzinger, 1976; Eapen et al., 1989; Jain et al., 1989; Mathur et al., 1988; Secor and Shepard, 1981). Many reports of somaclonal variation do not indicate the genetic basis of the observed changes, although some alterations of the genetic material have been documented among somaclones. Genetic changes may be demonstrated by inheritance of the altered character among the sexual progeny, as was shown for somaclones of oat (Cummings et al., 1976), barley (Breiman et al., 1987b), and tomato (Evans and Sharp, 1986a, 1986b). In other cases, cytological, biochemical, or molecular studies have revealed a more specific genetic basis for the somaclonal variation.

Type of change - polyploidy. Euploid increases in chromosome number among plants regenerated from tissue culture have been reported for many crops (D'Amato, 1977a, 1978), including bluestem grasses (Taliaferro et al., 1989), tobacco (Ogura, 1976), tall fescue (Eizenga, 1989), alfalfa (Saunders and Bingham, 1972) and wild rye (Park and Walton, 1989). Osifo et al. (1989) produced callus from the cotyledons of a diploid potato selection and reported that 70% of the regenerated somaclones were tetraploid. Hexaploid and octoploid plants were found among the regenerates from protoplasts of two tetraploid potato cultivars (Karp et al., 1982), while Singh et al. (1972) obtained haploid plants from cultures of a diploid legume species. However, examination of a large number of plants regenerated from haploid, diploid, and triploid lines of beet detected none with alterations in the original chromosome number (Detrez et al., 1989). Interspecific hybrids have been noted to yield polyploids after tissue culture (D'Amato, 1985); Cappadocia and Ramulu (1980) obtained 21 amphidiploids among 57 regenerated plants from the hybrid between two species of tomato.

Type of change - aneuploidy. Aneuploidy has been reported most frequently in somaclones from species that are polyploid (D'Amato, 1985); diploids are much more likely to become inviable as a result of loss or gain of one or a few chromosomes. Aneuploid somaclones have been identified in celery (Murata and Orton, 1983; Orton, 1983), alfalfa (Groose and Bingham, 1984; Johnson et al., 1984), tobacco (Ogura, 1976), potato (Karp et al., 1982), *Haworthia* (Ogihara, 1981) and many grasses (Ahoowalia, 1983; Eizenga, 1989; Reed and Conger, 1985; Taliaferro et al., 1989). Eizenga (1989) observed 59 aneuploids among the 166 tall fescue

somaclones she examined. Meiotic analysis of the tall fescue somaclones indicated that the aneuploids were monosomic, double monosomic, or triple monosomic but not nullisomic (Eizenga, 1989).

Type of change - gross chromosomal. Alterations in gross chromosomal structure, or macromutations, have been found among plants regenerated from tissue culture, as well as among in vivo sports (Peloquin, 1981). Chromosome translocations, inversions, duplications and/or deletions have been reported in somaclonal variants from oat (McCoy et al., 1982), ryegrass (Ahloowalia, 1978), potato (Shepard, 1982), maize (Benzion and Phillips, 1988; Lee and Phillips, 1987) and other species (Armstrong et al., 1983; Lapitan et al., 1984; Lee and Phillips, 1988). McCoy et al. (1978) noted the formation of tripolar divisions, ring chromosomes, and heteromorphic bivalents at meiosis of some plants from culture and considered non-homologous crossovers and deletions to be responsible for these abnormalities. Benzion and Phillips (1988) reported that over 12% of the somaclones regenerated from maize callus cultures during a 22 month period contained some type of cytological abnormality, and many of these were caused by the breakage of a chromosome between the centromere and a heterochromatic knob.

Type of change - simple genetic. Changes in one or a small number of genes have been identified in somaclones by segregation among progeny from controlled crosses in wheat (Larkin et al., 1984), maize (Edallo et al., 1981), rice (Sun et al., 1983; Fukui, 1983), soybean (Freytag et al., 1989), tobacco (Prat, 1983), lettuce (Engler and Grogan, 1984), and twelve different variants of tomato (Evans and Sharp, 1983). Shahin and Spivey (1986) produced protoplasts from a tomato cultivar susceptible to *Fusarium*

oxysporum f.sp. *lycopersici*, race 2, and reported the regeneration of plants resistant to the pathogen both with and without in vitro selection pressure. Segregation patterns among progeny of these tomato somaclones indicated that some of the somaclones were heterozygous and some homozygous for a single dominant resistance gene (Shahin and Spivey, 1986). Relatively minor genetic mutations in some somaclones have also been suggested by changes in isozyme banding patterns (Allichio et al., 1987; Jackson and Dale, 1989; Orton, 1983; Taliaferro et al., 1989) and restriction fragment length polymorphisms (Roth et al., 1989). One maize somaclone with a variant Adh1 allele was shown to have resulted from a single base substitution in the nuclear gene (Brettell et al., 1986a). Some cases of chromosomal mutations leading to expression of recessive genes may result from the genetic nullification of an obscuring dominant gene, rather than from the *de novo* generation of the recessive gene (Little, 1989).

Breiman et al. (1987a) reported changes in the number of rDNA spacers in somaclones from one line of wheat after only a few weeks of culture, and claimed that the studies of Appels and Dvorak (1982) and Saghai-Maroof et al. (1984) indicated equivalent changes should take many generations in vivo. Other workers have reported deficiencies in rRNA genes among somaclones from triticale (Brettell et al., 1986b) and potato (Landsmann and Uhrig, 1985).

Type of change - organelle DNA. Changes in mitochondrial DNA (mtDNA) have been observed in somaclones from maize (Gengenbach et al., 1981; Umbeck and Gengenbach, 1983), sugar beet (Brears et al., 1989), potato (Kemble and Shepard, 1984), and wheat (Hartmann et al., 1987, 1989;

Rode et al., 1987). Somaclones were recovered from Texas male sterile lines of maize (susceptible to *Helminthosporium maydis* race T) with male fertility or resistance to the toxin of *H. maydis* (both characters are controlled by mitochondrial gene[s]) (Brettell et al., 1980; Umbeck and Gengenbach, 1983). Brears et al. (1989) reported a mtDNA change in a sugar beet somaclone. The mtDNA change was a specific reversion of the RFLP pattern in the male sterile source genotype to the pattern of the normal (fertile) genotype, although the somaclone remained male sterile (Brears et al., 1989). Hartmann et al. (1989) examined the mitochondrial genome of wheat somaclones and also observed specific rearrangements to other known mitochondrial types, as well as some novel mitochondrial DNA patterns.

No rearrangements were observed in the chloroplast genome of sugar beet somaclones (Brears et al., 1986, 1989), and it generally has been considered that the chloroplast genome rarely undergoes rearrangement. However, Evans and Sharp (1986) reported identification of changes in chloroplast DNA among some regenerated tomato plants. In gametoclonal studies, regeneration of plants from anther culture was reported to yield selections with deletions from the chloroplast DNA (Day and Ellis, 1984).

Type of change - methylation. Alterations in the methylation of DNA have been reported among cultured plant cells (Anderson et al., 1990; Quemada et al., 1987). The level of DNA methylation has been suggested to influence or indicate the expression of some genes, with reduced methylation generally corresponding to greater expression (Amasino et al., 1984; Bird, 1986; Cedar, 1988; Flavell et al., 1986; Hepburn et al., 1983; Holliday, 1987, 1989; Jablonka and Lamb, 1989; Watson et al., 1987). The

observation that treatment of plant tissues with a demethylating agent, 5-azacytidine, increases expression of some genes (Amasino et al., 1984; Hepburn et al., 1983) has shown that reduced methylation may promote gene expression. However, the relative degrees of methylation for rRNA genes in shoots, roots, and callus cultures of petunia differed widely and were not correlated with relative gene expression or growth (Anderson et al., 1990). The fact that higher plants contain many copies of rRNA genes, and only a small portion of them are expressed at one time (Rogers and Bendich, 1987) may make methylation of rDNA a poor indicator of total relative gene expression. Quemada et al. (1987) suggested that changes in methylation may be a cause of habituation in cell cultures. Nevertheless, changes in gene methylation have been proposed as a possible source of morphological variation in plants regenerated from tissue culture (Brown and Lorz, 1986; Lee and Phillips, 1988; Quemada et al., 1987). Patterns of methylation have been suggested to be heritable (Holliday, 1989; Jablonka and Lamb, 1989), but it is unclear whether methylation is a determiner or a consequence of gene activity. Generally, ribosomal genes in petunia shoots were highly methylated, those in callus cultures were variable in the degree of methylation (although the level of methylation for each cell line remained stable over time), and regenerated shoots typically returned to the degree of methylation observed in the original explants (Anderson et al., 1990).

Source of variation - preexisting. Evidence for morphological and genetic variation among plants regenerated from tissue culture is abundant. However, at least a portion of this variation is derived directly, without change, from the genetic and heritable epigenetic

differences (methylation?) that are common among cells within a single plant (D'Amato, 1975, 1977a). Cells in meristems generally do not change in genetic composition over long periods of time (D'Amato, 1985), as demonstrated by the large number of species that have been propagated uniformly by meristem culture (Conger, 1981). However, many different tissues can be successfully used for culture initiation (Murashige, 1974), including differentiated tissue that does not have a high degree of genetic fidelity. Explants from non-meristematic parts of the plant have been considered most likely to yield variation in somaclones (Meins, 1983; Reisch, 1983). Nearly 90 percent of the flowering plant species studied have been found to exhibit some level of endoreduplication of the DNA within the cells from differentiated tissues (D'Amato, 1977b, 1985). This results in an elevated level of ploidy that may frequently vary over a wide range within a single tissue (2x, 4x, 8x, or even higher). Plants with differentiated tissues that are composed of a mixture of cells with different ploidy levels are called polysomatic (D'Amato, 1985). Replication of chromosomes without separation of the chromatids (endomitosis) may occur to form polytene chromosomes, as in the cotyledons of pea (Karp and Bright, 1985). Other species, such as *Crepis*, have been found to maintain the same level of ploidy throughout their meristematic and somatic tissues (Brossard, 1978).

Variation in chromosome numbers other than in genome sets (aneuploidy) is not common in vivo but has been observed in the meristems and throughout the tissues of several plant species (D'Amato, 1985). Vaarama (1949) studied the tremendous range in aneuploidy found in a

tetraploid black currant (4x=32). Within the root tips, cells could be identified containing from 4 to 32 chromosomes.

Sub-chromosomal nuclear and organellar variation also occurs in plant tissues undergoing differentiation (D'Amato, 1952, 1985), although it is frequently difficult to identify. Examples are generally limited to those that have a visual effect, such as the white and green leaf sectors that result from different chloroplast types (Poethig and Sussex, 1985). The color of stamen hairs in *Tradescantia* is controlled by a single gene that has been observed to mutate from heterozygous to homozygous recessive at a frequency greater than 0.04% under normal conditions (Dolezel and Novak, 1984). Some cases of variation in plants regenerated from culture have been directly related to the isolation of one or more layers from the chimeric source plant (Hall et al., 1986a, 1986b, 1986c; McPheeters and Skirvin, 1989; Skeene and Barlass, 1983). Chromosomal crossing-over in somatic tissues has been observed among several crop species (Evans and Paddock, 1976) and is a mechanism (as is sister chromatid exchange [see below]) by which genetic variation can be produced among the cells within an individual. Much of the variation in plants regenerated from pollen or cells undergoing meiosis (sometimes called gametoclonal variation) probably results largely from the segregation of chromosomes and organelles in the meiotic processes and is best considered as a different phenomenon than somaclonal variation, although the two have frequently been discussed together (Evans and Sharp, 1986a; Evans et al., 1984).

An indication of the significance of explant source tissues to variation obtained in culture can be found by comparing cell populations

or regenerates obtained from different tissues. Wardell and Skoog (1973) reported tobacco pith cells varied in their DNA content, depending upon the height in the plant where the samples were taken. Plants regenerated from the cells lower in the stem had a different morphology from those regenerated from cells collected at the higher levels. Osifo et al. (1989) observed a much greater frequency of polyploidy among potato somaclones regenerated from cotyledon-derived callus than those regenerated from leaf-derived callus. Feher et al. (1989) reported that the chromosome number of tetraploid alfalfa somaclones regenerated by embryogenesis was directly related to the chromosome number of the original explant. Explants composed of somatic cells with 30 chromosomes produced only somaclones with 30 chromosomes. Aneuploid explants with 29 chromosomes and euploids with 2n=32 resulted in somaclones with a diversity of chromosome numbers, including a high percentage of mixoploids (Feher et al., 1989). However, in some cases, even the presence of genetic diversity within explant tissues does not lead to genetic variation among regenerated plants. Polyploid cells are common in sugar beet leaf tissue, but no polyploid, aneuploid or chimeric plants were observed among a large number of somaclones regenerated via adventitious shoots from petiole tissue (Detrez et al., 1989). The authors suggested that this was the result of selection against polyploid cells during the formation of in vitro adventitious buds.

In some cases, the tissue culture process seems to select for genotypes that are rare in the explant tissue. Ramulu et al. (1989) examined the ploidy levels of somaclones regenerated from protoplasts of monohaploid, dihaploid, and diploid potato selections. No haploid and

only a low frequency of diploid somaclones were recovered from any of these sources. Most of the somaclones from all three ploidy sources were tetraploid (Ramulu et al., 1989). Haploid cultures of *Crepis* (Sacristan, 1971) and *Datura* (Furner et al., 1978) became diploid during extended periods of culture.

Source of variation - dedifferentiation. At least a part of the variation observed among plants obtained from tissue culture may be produced or stimulated by the tissue culture process itself. One indicator of this is the changes in chromosome number that have been reported to occur in plant cell cultures. Studies by Binarova and Dolezel (1988) indicated that there was a slight increase in the frequency of cells at higher ploidy during initiation of embryogenic alfalfa suspension cultures, but frequencies reverted to normal after a short period in culture. Similar types of changes during culture initiation (and dedifferentiation) have been reported for alfalfa (Feher et al., 1989), tobacco (Bennici and Caffaro, 1985), onion (Dolezel and Novak, 1985), and pine (Franklin et al., 1989). Furner et al. (1978) observed that polyploidy was produced by the stimulation of premitotic endoreduplication during the dedifferentiation process. Using explants with genetic markers, Barbier and Dulieu (1983) reported that most of the culture-induced changes occurred during the first few cell divisions. Cionini et al. (1978) studied the callus induction phase in broad bean, and observed that the DNA composition of cells sometimes underwent an irregular division process, called fragmentation. When mitosis and cytokinesis followed, the resulting cells exhibited various degrees of aneuploidy. Selective amplification of certain DNA sequences has been reported in one

tobacco species during initial phases of dedifferentiation (Durante et al., 1983). Such differential DNA replication may affect fragmentation, mutation, and gene expression.

Source of variation - extended culture. In addition to those changes induced during dedifferentiation, other variation may occur slowly over extended periods of culture (Barbier and Dulieu, 1983). Chromosome polyploidization may arise by endoreduplication (D'Amato, 1985), fusion of spindles in cells with two nuclei (Mitra and Steward, 1961), or spindle failure (Bayliss, 1973). An increase in the frequency of cells at higher ploidy levels commonly has been reported for long term cell cultures (Bayliss, 1980; Berlyn et al., 1986). A steady increase in ploidy level was observed for cell cultures of Coulter pine, so that six weeks after culture initiation about 80% of the cells had 4C DNA (diploid cells in G2, or tetraploid cells), and regenerated buds contained many cells at the 8C level (Patel and Berlyn, 1982). However, other studies with pine cultures have indicated relatively little change in cell ploidy over time (Franklin et al., 1989; Konar and Nagmani, 1972; Renfroe and Berlyn, 1984; Salmia, 1975), and there was no abnormality in the amount of DNA found in adventitious shoots regenerated from 3-6 month old spruce cultures (Hakman et al., 1984). Degree of aneuploidy may also increase in cultures with age. Murashige and Nakano (1965) noted the higher frequency of aneuploidy found in tobacco cultures as they grew older. Wheat somaclones obtained from long term cultures were reported to be more likely to have mitochondrial DNA rearrangements than plants obtained from short term cultures (Hartmann et al., 1989).

Some of the diversity in frequencies of somaclonal variation reported by researchers working on the same crop may be the result of specific differences in culture technique. Alfalfa plants regenerated from callus cultures were reported to be morphologically and karyologically normal by some workers (Binarova and Dolezel, 1988; Kao and Michayluk, 1980; Mezentsev, 1981), while other reports indicated considerable morphological and karyological deviance among somaclones (Groose and Bingham, 1984; Johnson et al., 1984; Nagarajan and Walton, 1987). Studies by Binarova and Dolezel (1988) indicated that if short subculture intervals were used for alfalfa suspension cultures, the normal ploidy level and the capacity for embryogenesis were maintained. When the period between subcultures was extended to greater than seven days, there was an increase in the frequency of polyploid cells in the cultures and a corresponding loss in embryogenic potential (Binarova and Dolezel, 1988). This may be related to the differentiation process that begins once cell cultures reach the stationary growth phase. Cell differentiation frequently includes cell wall thickening, vacuolization, and DNA endoreduplication (Kibler and Neumann, 1980).

Source of variation - hormones in medium. Hormone composition of the media was reported to influence the degree of endoreduplication in pea cortex cells during dedifferentiation (Libbenga and Torrey, 1973). Auxins like 2,4-D have been suggested to have an important effect on the induction and frequency of somaclonal variation, including ploidy level stability (Bayliss, 1980). Relatively high concentrations (at least 25 $mg \cdot l^{-1}$) of 2,4-D and 2,4,5-T (another auxin) were found to induce cytological abnormalities in onion root tips (Croker, 1953). Lazar et al.

(1983) observed that tissue culture medium with moderate levels of 2,4,5-T (1 $mg \cdot l^{-1}$) resulted in a higher frequency of aberrant plants than the same medium with a low 2,4,5-T concentration. However, Binarova and Dolezel (1988) noted considerable ploidy stability in alfalfa suspension cultures initiated on medium with a high level of 2,4-D, and other researchers have found that growth regulator components of the media had no effect on the karyological stability of cultures (Hanisch Ten Cate and Sree Ramulu, 1987). The relative ability of a chemical to induce sister chromatid exchanges (SCE) is considered to be a good indicator of mutagenic action (Latt, 1974; Perry and Evans, 1975; Uggla and Natarajan, 1982). Murata (1985, 1989) made use of this fact to examine the potential mutagenic effects of NAA, 2,4-D, 2,4,5,-T, and kinetin on wheat cell cultures. Concentrations of 2-5 $mg \cdot l^{-1}$ of 2,4,5-T significantly increased the frequency of SCE in the wheat cell cultures. However, NAA, 2,4-D, and kinetin were not observed to have any effect on the frequency of SCE, although inclusion of kinetin reduced the effects of 2,4,5-T.

Mechanisms for induction. One mechanism of somaclonal variation may be the formation of SCEs or some other types of mitotic recombination. Larkin and Scowcroft (1981) suggested that asymmetric crossing-over or recombination between homologous chromosomes could lead to some of the macromutations that had been observed in somaclones. Limited evidence for the occurrence of mitotic recombination among plants regenerated from tissue culture has been presented by several authors (Barbier and Dulieu, 1983; Dulieu and Barbier, 1982; Larkin et al., 1984; Lorz and Scowcroft, 1983; McCoy, 1980).

Chromosome fragmentation among plant cell cultures and somaclones has been observed to commonly occur in heterochromatic regions (Armstrong et al., 1983; Brettell et al., 1986; Lapitan et al., 1984; McCoy et al., 1982; Murata and Orton, 1984) or near the nucleolus organizer region (Lee and Phillips, 1987; Sacristan, 1971). Heterochromatin is known to replicate late in the mitotic cycle (Lima-De-Faria, 1969), so the formation of a bridge in the heterochromatic region and subsequent chromosome breakage has been proposed as one mechanism of somaclonal variation (Benzion and Phillips, 1988; Johnson et al., 1987; Lee and Phillips, 1987, 1988; McCoy et al., 1982; Sacristan, 1971).

Nucleotide pool imbalances may produce genetic changes in unicellular organisms (Kunz, 1982; Kunz and Haynes, 1982) and animals (Weinberg et al., 1981), including SCEs, aneuploidy, and macromutations in the nuclear and organellar genomes. Lee and Phillips (1988) have proposed that cell culture may cause critical imbalances in the nucleotide pools of plant cells that lead to similar genetic changes, and thus somaclonal variation.

Another mechanism that has been suggested for the induction of somaclonal variation is the activation of transposable elements by stresses on the plant genome resulting from the tissue culture process (Chaleff, 1983; Evola et al., 1985; Groose and Bingham, 1986a, 1986b; Larkin and Scowcroft, 1981; Peschke et al., 1987) or some other specific stress-induced rearrangement process (Benzion et al., 1986; Brettell et al., 1986b; Murata and Orton, 1983). Roth et al. (1989) suggested that the RFLP changes observed in inbred soybean suspension cultures may have been caused by a mechanism that evolved to produce genetic variation in

response to stress. The changes that were observed at all loci were almost entirely alternative alleles found in other soybean cultivars. It was suggested that this may indicate that a specific recombinational or rearrangement event occurred, such as gene conversion, transposon movement, or inversion. Equivalent programmed gene rearrangements have been documented in mammals and some unicellular organisms (Borst and Greaves, 1987). Breiman et al. (1987a) proposed that the alterations they observed in intergenic spacers of regenerated somaclones were the result of tissue culture stress disrupting the mechanisms that normally maintain the stability of repeated DNA sequences. It is thought that repeated sequences play an important role in the adaption of the genome to change (Flavell, 1985). Peschke et al. (1987) initiated cultures from maize explants without transposable element activity and reported the recovery of active Ac transposable elements in regenerated plants. Transposable elements have been best studied in maize, but are found in many other plant species as well (Grandbastien et al., 1989; Groose and Bingham, 1986a, 1986b). Maize transposable elements can be activated by different types of shocks or stresses to the genome (McClintock, 1978, 1984), such as chromosome breakage (Freeling, 1984), chromosome rearrangements (Freeling, 1984; McClintock, 1978; Peterson, 1986), and alterations in DNA methylation (Chandler and Walbot, 1986). All three of these processes occur in plant tissue cultures (see above).

Differences between reports. Frequency of variants obtained from culture has varied widely in the different cases where it has been examined. Daylily has been observed to produce little somaclonal variation (Krikorian et al., 1981), and Griesbach (1989) was able to

identify only one stable variant among 1000 daylily somaclones regenerated by organogenesis from long-term callus cultures. In contrast, Eapen et al. (1989) reported alterations in mustard plant height, pod number, seed weight per plant, and yield in the second generation of nearly all plants regenerated from protoplasts when compared with the parental genotype. Frequently different researchers report entirely opposite results for the same crop and similar methods. Secor and Shepard (1981) examined 65 protoplast-derived somaclones of potato and determined that every one was statistically different from the source cultivar in at least one of the 35 characteristics measured (the authors indicated that this observation was quite significant, but the statistical significance is questionable), while a previous study (Wenzel et al., 1979) reported that there was no variation among protoplast-derived potato plants. Lourens and Martin (1987) observed that morphological variability among sugarcane somaclones was much less than previously reported (Heinz et al., 1977; Heinz and Mee, 1971; Heinz et al., 1969) when deviant somaclones were vegetatively propagated and examined after a second cycle of growth. Some of the discrepancies within the literature may be caused by confusion between temporary epigenetic changes and permanent genetic mutations. In addition, kinds and amount of variation are dependent upon the characters measured (Daub and Jenns, 1989). Three somaclonal variants for gliadin proteins in wheat (Maddock et al., 1985) were not observed to be deviant when examined for a variety of morphological characteristics (Maddock and Semple, 1986).

In other cases, dissimilar results may be associated with large differences between species, or between cultivars within a species, in

response to tissue culture (Bright and Jones, 1985) and in the frequency of somaclonal variation (Breiman et al., 1987; Brettell et al., 1986; Karp and Bright, 1985). Maddock and Semple (1986) speculated that the much lower frequency of somaclonal variation they observed (Maddock et al., 1985; Maddock and Semple, 1986) than Larkin et al. (1984) among regenerated wheat plants was caused, at least partly, by cultivar differences. Binarova and Dolezel (1988) reported a high degree of karyological stability and maintenance of embryogenic capacity for one line of alfalfa in suspension culture, while Atanasov and Brown (1984) observed a considerable shift in cell ploidy and loss of embryogenesis in a second line maintained under similar conditions.

Method of regeneration may also affect the frequency of variation among somaclones. Regeneration by adventitious shoots usually has produced a greater frequency of somaclonal variants than regeneration from axillary meristems (Conger, 1981; Larkin and Scowcroft, 1981). Organogenic regeneration generally has been considered to result in more somaclonal variation than embryogenesis (Armstrong and Phillips, 1988; Vasil, 1987), although this has not always proven true. Regeneration by organogenesis from long-term callus cultures resulted in very little variation in daylily (Griesbach, 1989), while regeneration of mustard plants by direct somatic embryogenesis was reported to produce over 95% somaclonal variants (Eapen et al., 1989). A study comparing the variation among maize plants regenerated by the two methods indicated that the frequency of variation was great for plants from embryogenesis, but even greater for those from organogenesis (Armstrong and Phillips, 1988). The process of plant regeneration may apply some selection against genetically

aberrant cell types that survive in culture but are not viable in vivo (D'Amato, 1985; Vasil, 1988). Feher et al. (1989) observed that embryogenesis occurred normally in alfalfa cultures containing high percentages of aneuploid and/or polyploid cells, but that the resulting embryos were mostly of the normal chromosome number. Polyploid cells are common in sugar beet leaf tissue, but no polyploid, aneuploid or chimeric plants were observed among a large number of somaclones regenerated by adventitious shoots from petiole tissue (Detrez et al., 1989); the authors suggested that this was the result of selection against polyploid cells during the formation of in vitro adventitious buds.

As mentioned above, culture age may also affect variation among regenerated plants. The frequency of somaclonal variation typically has been observed to increase with aging of the callus from which plants are regenerated (Armstrong and Phillips, 1988; Lee and Phillips, 1987). No meiotic aberrations were observed among maize somaclones regenerated from cultures 3-4 months old, but nearly half of the somaclones regenerated from similar cultures 8-9 months old contained cytological aberrations (Lee and Phillips, 1987). Benzion and Phillips (1988) presented evidence that the increased frequency of cytogenetically abnormal maize somaclones from older cultures was "due to mutational events that occurred [at a constant rate] throughout culture development with a subsequent maintenance and accumulation of aberrant cells over time" (p. 318). However, callus age has not always resulted in a high frequency of variants; daylily callus more than 20 months old produced few somaclonal variants (Griesbach, 1989).

Value. Despite numerous reports of interesting or potentially useful mutants obtained by somaclonal variation, relatively little benefit has been evidenced in commercial varieties or breeding lines. One problem with some clones derived by tissue cultures, especially those derived by in vitro selection, is that what appear to be favorable changes in cell characteristics or even greenhouse plant response may not translate into improved field performance. A salt-tolerant somaclone of flax obtained by in vitro selection (McHughen and Swartz, 1984) was shown to be more productive than its parent cultivar under greenhouse conditions (McHughen, 1987). However, extensive field testing at both saline and nonsaline sites did not indicate that the salt-tolerant line was more productive than the parent cultivar under either condition (Rowland et al., 1988, 1989). In the case of sugarcane, it has been suggested that the tremendous amount of variation already present in the germplasm renders the minor variation obtained from culture relatively insignificant (Maretzki, 1987). Another limitation is that mutations produced by somaclonal variation are random and, like all types of random mutations, are almost always deleterious or undesirable (Micke et al., 1987; Stadler, 1928a, 1928b).

The novelty of the mutations obtained determines the relative value of any mutagenic method, including somaclonal variation. Some novel mutations have been reported among somaclones (Evans, 1989; Evans et al., 1989; Griesbach, 1989). However, the types of variation in potato somaclones (Sanford et al., 1984), tobacco somaclones (Daub and Jenns, 1989) and wheat somaclones (Maddock and Semple, 1986) were not found to be different from those that occurred naturally under field conditions or

were already known in germplasm collections. Sanford et al. (1984) pointed out that sub-clonal selection in the field had been used for potato improvement (Davidson and Lawley, 1953; Easton and Nagle, 1981; McIntosh, 1945) but was generally proven less effective than sexual hybridization. Gavazzi et al. (1987) compared tomato mutants derived from somaclonal variation with those produced by ethyl methane sulphonate (EMS) treatment in vivo, and determined that somaclonal variation produced a higher frequency of variants. In addition, the types of mutations produced by somaclonal variation and EMS methods were different; *Verticillium* resistance was only recovered from EMS treatments (Gavazzi et al., 1987). Montagno et al. (1989) compared somaclonal variation in tomato with that induced by mutation with ionizing radiation. The two methods produced similar types of mutations, although radiation (at 6.5 krad) produced heritable changes at more than twice the frequency of somaclonal variation.

There is little doubt that somaclonal variation does occur among regenerates from many tissue culture systems. However, the ultimate value of somaclonal variation (or any method of genetic improvement) is determined by the relative expense (time and money) incurred and the relative genetic advancement achieved. Maddock and Semple (1986) have aptly pointed out that "where genetic variation *per se* can be obtained readily by standard sexual crossing procedures, random tissue culture-induced variability may, therefore, not be of great value if large-scale screening of plant populations is necessary" (p. 1076). The relative value of somaclonal variation increases when sexual hybridization is difficult or concurrent in vitro selection is possible.

Somaclonal variation in *Citrus*. Somaclonal variation in plants regenerated by embryogenesis from *Citrus* has been evaluated by several researchers (Gmitter, 1985; Kobayashi, 1987; Navarro et al., 1979, 1985; Vardi, 1977; Vardi et al., 1982) with somewhat inconclusive results. One of nine plants regenerated from embryogenic orange cultures by Vardi (1977) was tetraploid, but the author did not indicate whether this plant was from normal or X-irradiated cultures. In later studies, Vardi et al. (1982) reported that nucellar callus lines from seven *Citrus* cultivars maintained normal chromosome numbers, while an eighth nucellar callus line from 'Villafranca' lemon (*C. limon* [L.] Burm.) became predominantly tetraploid. Vardi et al. (1982) indicated that plants with normal morphology were regenerated from each of these callus cultures. Navarro et al. (1979) reported that regenerated sweet orange (polyembryonic) somaclones were morphologically normal. In contrast, Navarro et al. (1985) identified about 28% of the somaclones as morphologically abnormal after regeneration from nucellus explants of 'Clementine' (a monoembryonic cultivar) by somatic embryogenesis. The observation of uniformity among somaclones obtained from the same explant in this latter study (Navarro et al., 1985) may indicate that the variation was preexistent in the explant and was not the result of the tissue culture process. Kobayashi (1987) examined morphological, cytological, and biochemical characteristics of 25 orange somaclones regenerated by embryogenesis from protoplasts and determined that they were "identical" to the nucellar seedlings. Gmitter (1985) identified morphologically abnormal somaclones among the plants regenerated by embryogenesis, but did not observe any

cytological or isozymic variation. No published studies have systematically examined *Citrus* populations regenerated via organogenesis.

In Vitro Mutagenesis by Irradiation

Mutation was first employed as a method of genetic improvement over sixty years ago (Muller, 1927; Stadler, 1928a, 1928b). Induced mutations do not necessarily duplicate natural genetic variation (Allard, 1960; Herskowitz, 1962; Stubbe, 1967). Most mutants will be lethal or unusable because they are clearly deleterious (Brock, 1971; Hansel, 1967). However, this has not prevented the utilization of mutagenesis as a breeding strategy; by the end of 1986, over 700 cultivars that were developed by mutation breeding had been released (Gottschalk and Wolff, 1983; Konzak, 1984; Micke and Donini, 1982; Micke et al., 1985; Micke et al., 1987). Mutation in plant materials has most commonly been induced by chemical mutagens or radiation. Chemical mutagens were not utilized in this dissertation research (unless one considers 2,4-D as a mutagen; see above), but the types and usage have been discussed by several authors (IAEA, 1977; Kleinhofs et al., 1974; Levy and Ashri, 1975; Moustafa et al., 1989; Neale, 1976).

Radiation has many different effects on living tissues (Coggle, 1983; Dertinger and Jung, 1970; Wierbicki et al., 1986), and one of these effects is the induction of genetic mutations (Coggle, 1983; Lea, 1955; Savage, 1989; United Nations, 1986). High energy forms of radiation, including X- and gamma-rays, are typically known as ionizing radiation because of their ability to cause electrons to be driven from atoms which they contact. Both X- and gamma radiation have been investigated as

methods of inducing mutations for crop genetic improvement (Broertjes, 1982; Eapen, 1976; Hearn, 1984, 1986; Hensz, 1977; Huitema et al., 1989; Liu and Deng, 1985; Moustafa et al., 1989; Russo et al., 1981; Sharma et al., 1989; Van Harten et al., 1981; Wang et al., 1988; Werry, 1981). The mutagenic effects of ionizing radiation on plant cells have been studied by cytology (Kuehnert, 1962), morphology (Alexander et al., 1971; Kuehnert, 1962; Yu and Yeager, 1960), and isozymes (Gulin et al., 1989), and were quantified by the measurement of sister chromatid exchanges (Kuglik et al., 1987, 1989). Some mechanisms of gamma-induced mutation in plants may be similar to those described for yeast (Baranowska et al., 1987; Chepurnoi, et al., 1989) or may involve the induction of double stranded breaks and imperfect repair (Charlton et al., 1989). Irradiation for induction of mutations can be easily applied to plant material in vivo, but the resulting mutations have been frequently chimeric, complicating their identification and isolation (Broertjes, 1982; D'Amato, 1965). Irradiation of callus cultures may avoid these problems because embryos or adventitious shoots regenerated from tissue cultures often develop from single cells (Bhatia et al., 1986; Broertjes et al., 1968; Broertjes and Keen, 1980; Micke et al., 1987; Van Harten et al., 1981). The special advantages of in vitro mutagenesis when it can be used in combination with in vitro selection have been noted (Chaleff, 1981; Huitema et al., 1989; IAEA, 1985; Ingram, 1983; Ingram and MacDonald, 1986; Werry, 1981), although unsolved problems (such as identifying in vitro selection methods that correlate with field responses) have generally remained obstacles to the successful utilization of these techniques (Lorz and Brown, 1986; Wersuhn, 1988, 1989).

Low levels of ionizing radiation sometimes have been reported to stimulate plant growth or morphogenesis, a phenomenon termed radiation hormesis (Hell, 1983; Miller and Miller, 1987; Nuttall et al., 1968; Sagan, 1989; Sax, 1963; Sheppard and Chubey, 1990; Sidrak and Suess, 1973). This effect has not been observed in other cases after the same levels of irradiation (Montagno et al., 1989; Wolff, 1989). Whether the phenomenon of hormesis has any bearing on mutation induction is unclear.

Sensitivity to irradiation has been observed to vary by a factor of up to 500 among different species (Balito et al., 1989; Sparrow and Woodwell, 1962), and differences also have been reported between tissues and organs of a single species (Bajaj et al., 1970). Large nuclear volumes and rapid growth rates generally correlate with greater radiosensitivity, although higher ploidy levels tend to be more resistant (Balito et al., 1989; Eapen, 1976; Galun and Raveh, 1975; Sparrow et al., 1961; Sparrow and Woodwell, 1962). Gamma irradiation of tissue cultures for genetic improvement has been investigated in several crops (Broertjes, 1982; Eapen, 1976; Moustafa et al., 1989), although as Balito et al. (1989) noted, only limited information is available on the effects of gamma irradiation on plant tissue cultures or regenerated plants. Moustafa et al. (1989) observed that regeneration of maize plants from callus was considerably more sensitive to gamma irradiation than was the further growth of the callus. A dose of 100 Gy gamma radiation reduced maize callus growth about 50%, while a similar reduction in plant regeneration occurred with a treatment of only 25 Gy.

Clones with increased flesh and rind color, fewer seeds, and decreased flesh acidity have been obtained by irradiation of *Citrus*

budwood or nucellar seeds (Hearn, 1984, 1986; Hensz, 1971, 1977, 1985; Russo et al., 1981; Spiegel-Roy and Kochba, 1973a; Spiegel-Roy et al., 1985; Starrantino et al., 1988a, 1988b; Yen, 1987; Zubrzycki and Diamante de Zubrzycki, 1982). Despite the striking achievements of in vivo irradiation relative to other approaches to *Citrus* genetic improvement, only limited effort has been devoted to irradiation of *Citrus* tissue cultures (Chang et al., 1984; Kochba and Spiegel-Roy, 1982; Legave et al., 1989; Liu and Deng, 1985; Spiegel-Roy and Kochba, 1973b; Vardi, 1977). A dramatic stimulation of *Citrus* embryo formation from embryogenic cultures after gamma or X-irradiation has been reported by several workers (Nito et al., 1989; Spiegel-Roy and Kochba, 1973b; Vardi, 1977). Liu and Deng (1985; Chang et al., 1984) treated organogenic callus cultures of several sweet orange cultivars with gamma radiation from a Cobalt-60 source. The LD-50 dose was determined to be 6-7 krad by percentage of callus survival, and gamma irradiated callus was noted to have a reduction in bud formation and bud growth when compared to controls. Increased frequencies of chromosome aberrations and polyploids were observed among the cells of the irradiated callus (Liu and Deng, 1985). Vardi (1977) reported an LD-50 of 3.4 krad X-radiation for sweet orange protoplasts and obtained several plants from the irradiated callus, including one tetraploid. Nito et al. (1989) studied the effect of gamma radiation on callus growth and embryoid formation in cultures of 'Valencia' (*Citrus sinensis* [L.] Osbeck), 'Yoshida' (*C. sinensis*), Calamondin (*C. madurensis* Lour.), Yuzu (*C. junos* Sieb. ex Tan.), and 'Ishizuka Wase' satsuma (*C. unshiu* Marc.), and reported LD-50 values of 10-20, 20-50, 20-50, 1-5, and 10-20 krad, respectively. These LD-50 values are similar to those

reported by Spiegel-Roy and Kochba (1973b), but are difficult to reconcile with the values of 3-7 krad reported by other researchers (although a difference between embryogenic and organogenic cultures may contribute) for irradiation of *Citrus* callus cultures (Chang et al., 1984; Legave et al., 1989; Liu and Deng, 1985; Vardi, 1977).

Fruit Sector Chimeras

A chimera may be defined as an organism or organ containing two or more genetically distinct tissue types (Cramer, 1954; Tilney-Bassett, 1986; Vaughn, 1983). Chimeras have been described in many plant species (Chevreau et al., 1989; Pratt, 1983; Tilney-Bassett, 1986; Vaughn, 1983) and have been classified in a number of different ways. Although normal grafted plants are not considered to be chimeras because the two cell types are completely separated except for the graft union (Cameron and Frost, 1968; Neilson-Jones, 1969), chimeras may develop from grafted plants when the two dissimilar genotypes grow together and produce "hybrid" organs containing intermingled cells of both genotypes (generally segregated by histogenic layers).

Most species of dicotyledonous plants (including *Citrus* [Cameron and Frost, 1968]) behave as though there are three histogenic layers in the apical meristem (Gifford, 1954). The two layers of the tunica (the L-I and L-II) generally form the epidermal and subepidermal (including gametophytic and nucellar) tissues, respectively (Pratt, 1983). The innermost layer of the apical meristem is called the corpus (L-III) and produces the central tissues of most organs including much of the vascular system (Pratt, 1983). The fruit of *Citrus* spp. are rather unusual in that

the rind is derived predominantly from the L-II layer, but the flesh (modified epidermal hairs) is derived mostly from L-I (Cameron and Frost, 1968; Cameron et al., 1964).

Chimeras produced by grafting are termed graft chimeras, or synthetic chimeras (T. Tanaka, 1927b; Winkler, 1907), and may be distinguished from those produced by nature, called autogenous (Cameron and Frost, 1968; Neilson-Jones, 1969). Natural chimeras are sometimes further classified according to type of genetic difference. Cytochimeras, or chromosomal chimeras, are composed of tissues of different ploidys (e.g. diploid and tetraploid) (Dermen, 1947; Einset et al., 1947; Marks, 1953) or distinct cell karyotypes (Brumfield, 1943), while genic chimeras are composed of tissues with relatively simple genetic differences (Chevreau et al., 1989; Dayton, 1966; Einset and Pratt, 1959). Spatial distribution in the plant tissues (i.e. histogenic layer origin) is perhaps the most common method of classifying chimeras (Swanson, 1957). A periclinal chimera is formed when genetically different cell types are completely segregated by histogenic layers (e.g. L-I, L-II, or L-III) and all of each layer is of a single cell type (Dermen, 1960; Doodeman and Bianchi, 1985; Hall et al., 1986a, 1986b; Jones, 1934; Marcotrigiano, 1986; T. Tanaka, 1927b). A chimera composed of a sector of tissue including a portion of all histogenic layers is termed a sectorial chimera (Dermen, 1947; Neilson-Jones, 1969; Varghese and Robbelen, 1984). The mericlinal chimera is distinguished from the other two types by the alternate genotype being found only in a part of one cell layer (usually the epidermis) (Jorgensen and Crane, 1927; Vaughn and Wilson, 1980). Chimeras composed of irregular combinations of the above types are known

as mixed or mosaic chimeras (Cameron and Frost, 1968). Chimeras, especially periclinal chimeras, of some plant species have been separated into the component genotypes as non-chimeric plants by irradiation (Johnson, 1980; Pereau-Leroy, 1974; Sagawa and Mehlquist, 1957), tissue culture (Dommergues and Gillot, 1973; Johnson, 1980), root cuttings (Decourtye, 1987), or production of adventitious shoots (Dommergues and Gillot, 1973; Stewart and Dermen, 1970).

Chimeras are common in *Citrus*, and one of the first plant chimeras ever described was the "bizzarria," a synthetic chimera produced by a failed graft of citron (*C. medica* L.) and sour orange (*C. aurantium* L.) about 1644 (Darwin, 1921; Gallesio, 1811; Nati, 1674, 1929; Risso and Poiteau, 1818; T. Tanaka, 1927b). Bud sports have occurred frequently on many *Citrus* cultivars (Bono et al., 1981; Devaux, 1981; Hensz, 1981; Iwamasa and Nishiura, 1981; Iwamasa et al., 1981; Mendel, 1981; Russo, 1981; Shamel, 1943; Soost et al., 1961) and probably originate as periclinal, sectorial or mericlinal chimeras. Mendel (1981) suggested "that varieties showing a tendency to produce bud mutations are chimeras themselves (e.g. the 'Shamouti' orange)" (p. 86).

Many *Citrus* cultivars are known to be relatively stable periclinal chimeras (Cameron and Frost, 1968; Cameron et al., 1964; Iwamasa and Nishiura, 1970). The pink-fleshed grapefruit 'Thompson' arose as a bud sport on a tree of the white-fleshed grapefruit, 'Marsh' (Hodgson, 1967). The cultivar 'Thompson' contains pink flesh (derived from L-I) but unpigmented rind (derived from L-II), while a budsport derived from it, 'Ruby Red', has both red flesh and pigmented rind. 'Thompson' nucellar seedlings (derived from L-II) produce only fruit with white rind and white

flesh. Nucellar seedlings from 'Ruby Red' produce only red-fleshed fruit with pigmented rind. 'Thompson' is probably a periclinal chimera containing a genetic mutation for red pigmentation in the L-I layer. 'Ruby Red' is nonchimeric for the red pigmented cell type and probably arose as a layer substitution in 'Thompson' (Cameron et al., 1964; Soost and Cameron, 1975).

Other *Citrus* chimeras that have been described include, 'Burgundy' grapefruit (Olson et al., 1966), 'Foster' grapefruit (Cameron et al., 1964), 'Golden Buckeye' navel orange (Shamel et al., 1925), 'Kobayashi Mikan' (Cameron and Frost, 1968; Iwamasa et al., 1977; Samura and Nakahara, 1928), 'Gailiangcheng' orange (Lu, 1978, 1982), 'Suzuki Wase' satsuma (Iwamasa and Nishiura, 1970; Iwamasa et al., 1977), and several variegated chimeras with white and green leaves (Cameron and Frost, 1968; Shamel, 1932). Nishiura and Iwamasa (1970) suggested that recovery of two color forms among nucellar seedlings of the satsuma selection 'Dobashibeni Unshu' may indicate that it is a sectorial chimera composed of orange-yellow and red pigmented components. *Citrus* cytochimeras have been observed with diploid, tetraploid, and octoploid layers (Barrett, 1974; Frost and Krug, 1942).

Fruit sector chimeras with changes in rind color (Cameron and Frost, 1968; Coit, 1915; Frost, 1926; Iwamasa et al., 1977; Nishiura and Iwamasa, 1970), rind thickness (Cameron and Frost, 1968; Coit, 1915; Frost, 1926; Shamel et al., 1918), rind texture (Cameron and Frost, 1968; Shamel et al., 1918), flesh color (Cameron and Frost, 1968), ripening season (Coit, 1915), or rind injury resistance (W. Grierson, personal communication) have been observed from trees of many *Citrus* cultivars; sometimes

individual trees produced the chimeras at a high frequency (Frost, 1926). Some 'Ruby' orange fruit with sectors having altered rind color were noted to contain adjacent pulp segments with similar color changes (Cameron and Frost, 1968). Coit (1915) noted that citrus workers commonly considered the sectored fruit to be the result of cross-pollination, but that such fruit were actually the result of a mutation in the tree or at the base of the ovary. Frost (1926) reported that the cause of some sectorial chimeras appeared to be "differential mitosis" or chromosome non-disjunction because occasionally chimeric fruit had "two adjacent sectors, of similar width, whose rind varies in opposite directions from the normal condition" (p. 394). Nishiura and Iwamasa (1970) suggested that the fruit sector chimeras observed on 'Dobashibeni Unshu' were an indication that this particular selection of satsuma was a sectorial chimera. The frequency of fruit chimeras with one or more thickened rind sectors, described as "ridging" or "coxcombing," was observed to be about 0.1% in navel orange, 0.1% in 'Valencia' orange, 0.2% for lemon, and 0.2% for grapefruit in normal California orchards (Sinclair and Lindgren, 1943). However, fumigation of *Citrus* trees with hydrocyanic acid (Lindgren and Sinclair, 1941; Sinclair and Lindgren, 1943) or chlorpyrifos (M.L. Arpaia, personal communication) during the early stages of fruit bud development (January or February) was found to cause a dramatic increase in the frequency of ridged fruit. Sinclair and Lindgren (1943) suggested that hydrocyanic acid fumigation may cause "a genetic change, such as a doubling of the number of chromosomes" (p. 104), in one or a small number of cells in the developing ovary, and that this mutation could then be propagated by mitosis to form the ridged sectors. Iwamasa et al. (1977)

isolated seeds from beneath the normal orange and mutant yellow sectors of one chimeric fruit of 'Fukuhara' orange. Trees that developed from seed borne in the orange sector produced unsectored orange-colored fruit, while those that developed from seed borne in the yellow sector produced unsectored yellow fruit. It was stated that a similar event had been previously observed by Iwamasa and Nishiura (1970). Iwamasa et al. (1977) suggested that the nucellar seed and rind sectors had developed from the same L-II histogen and that sectors with improved fruit color might therefore be used to produce better cultivars.

Evaluation of Resistance to Phytophthora

Phytophthora spp. cause serious soilborne diseases of many important world crops. Several *Phytophthora* species can invade *Citrus*, including *P. parasitica* Dast., *P. citrophthora* (R.E. Sm.& E.H. Sm.) Leonian, *P. hibernalis* Carne, *P. syringae* Kleb., *P. palmivora* (Butler), and *P. citricola* Saw. The most important of these species in Florida is *P. parasitica*, which is responsible for root rot, foot rot, and gummosis (Timmer and Menge, 1988), as well as causing damping-off of young *Citrus* seedlings (Timmer, 1988). *Citrus* feeder roots are invaded principally by zoospores from infected soil through wounds or undamaged root tips. Zoospore germination and mycelial growth lead to a subsequent decay of the root cortex that may result in tree decline (Timmer and Menge, 1988). *Phytophthora* can invade the trunk of *Citrus* trees through openings in the bark produced by wounding or natural cracking. The ensuing foot rot or gummosis is characterized by necrosis of the cambium and rotting of the bark. Foot rot is the name applied when the disease affects the tree

slightly above, at, or slightly below ground level, while the term gummosis is more frequently used when the disease occurs higher in the trunk. Trees affected by foot rot/gummosis may show symptoms of gum exudation, dieback, weak growth, and/or death (Lutz and Menge, 1986; Timmer and Menge, 1988).

Phytophthora can be controlled in the nursery by hot water treatment of seeds, fumigation of soil, or soil drenches with appropriate fungicides (Timmer and Menge, 1988). Some degree of control in young and mature orchards is possible by fungicide application as foliar sprays of systemic compounds (Davis, 1981; Sandler et al., 1989), drenches (for root rot), or trunk paints (for foot rot and gummosis), as well as by sanitation (Sandler et al., 1989; Timmer, 1977; Timmer and Menge, 1988). The most economical control is planting resistant rootstocks with the bud union well above soil level. Cultivars used as rootstocks vary widely in their susceptibility to the diseases caused by *Phytophthora* (Broadbent et al., 1971; Carpenter and Furr, 1962; Graham, in press; Hutchison and Grimm, 1972; Klotz et al., 1968; Klotz and Calavan, 1978; Smith et al., 1987; Timmer and Menge, 1988). Although a general correlation between relative resistance to root rot and foot rot has been observed for many *Citrus* selections, there are reports of substantial differences in response of some selections to the two diseases (Carpenter and Furr, 1962; Furr and Carpenter, 1961; Grimm and Hutchison, 1973, 1977). 'Carrizo' citrange and sour orange are tolerant to foot rot (Castle et al., 1989; Timmer and Menge, 1988), yet were reported to be susceptible to root rot (Graham, in press). Relatively little is known about the mechanism(s) of resistance to *Phytophthora* in *Citrus*, although the involvement of coumarin

phytoalexins has been suggested (Afek and Sztejnberg, 1986, 1988; Afek et al., 1986; Vernenghi et al., 1987). Isolate specificity has not been reported for *Phytophthora* within *Citrus*. However, Matheron and Matejka (1990) determined that isolates of *P. parasitica* isolated from tomato, petunia, and five other unrelated hosts were not virulent on rough lemon (*Citrus jambhiri* Lush.), and similar species specificity for isolates was observed by other workers (Wheeler and Boyle, 1971). Hutchison (1985) reported that resistance to *P. parasitica* in *Citrus* is probably controlled by a multiple gene system. Because of the great differences in characteristics of the affected tissues and etiologies (Lutz and Menge, 1986; Timmer and Menge, 1988), it seems probable that one or more mechanism(s) of resistance are not common to both root rot and foot rot.

Several methods of evaluating resistance of young *Citrus* to root rot and foot rot have been described. Soil flooding with zoospore suspensions has frequently been used to evaluate susceptibility to root rot (Cameron et al., 1972; Carpenter and Furr, 1962; Grimm and Hutchison, 1973; Whiteside, 1974), although the results have not always been reliable (Carpenter and Furr, 1962; Furr and Carpenter, 1961; Grimm and Hutchison, 1973). Tsao and Garber (1960) reported that soil infestation with mycelium grown in liquid cultures produced reliable results. Chlamydospores may provide a more uniform source of inoculum (Farih et al., 1981; Graham and Egel, 1988), and Graham (in press) described a procedure for chlamydospore inoculation and susceptibility assay.

Controlled evaluations of foot rot/gummosis resistance have typically been completed by wounding trunk tissue and inoculating with agar disks from mycelial cultures (Klotz et al., 1968; Smith et al.,

1987). General ratings of field resistance have commonly relied upon observations of foot rot/gummosis susceptibility because it is the most visible *Phytophthora* disease, despite a high degree of variability in damage observed at different times of testing (Smith et al., 1987; Whiteside, 1971).

Although several different methods of evaluating susceptibility are available, screening *Citrus* plants for resistance to *Phytophthora* has proven to be time-consuming and relatively imprecise in rootstock development programs (Broadbent, 1971; Carpenter and Furr, 1962; Furr and Carpenter, 1961; Grimm and Hutchison, 1973; Hutchison, 1985; Smith et al., 1987). The development of a more rapid and reliable method for determination of *Phytophthora* resistance would be of great benefit to breeding programs.

Tissue culture techniques have become increasingly important for programs involved in the genetic improvement of *Citrus* (Gmitter and Moore, 1986; Grosser and Gmitter, 1989; Grosser et al., 1988a, 1988b, 1989; Hidaka and Kajiura, 1988; Kobayashi and Ohgawara, 1988; Kobayashi et al., 1983, 1988; Kochba and Spiegel-Roy, 1982; Kochba et al., 1972; Navarro et al., 1985; Ohgawara et al., 1985; Sim et al., 1988; Vardi and Galun, 1988; Vardi et al., 1986b, 1987, 1989). Some success has been achieved in characterizing or selecting for plant resistance to diseases in vitro (Behnke, 1979, 1980; Brisset et al., 1988; Buiatti and Scala, 1985; Chang et al., 1989; Dunbar and Stephens, 1989; Wenzel et al., 1985; Willmot et al., 1989), and the advantages of in vitro selection have been noted (Chaleff, 1981, 1983; Huitema et al., 1989; IAEA, 1985; Ingram, 1983; Ingram and MacDonald, 1986; Werry, 1981). In vitro resistance of plant

tissues to *Phytophthora* in dual cultures (plant tissue and the pathogen together in axenic culture) has been correlated with field performance for: avocado (Dolan and Coffey, 1986; Zilberstein and Pinkas, 1987) and crops of seven other genera (McComb et al., 1987) to *P. cinnamomi* Rands; potato (Ingram, 1967) and tomato (Warren and Routley, 1970) to *P. infestans* (Mont.)DBy.; alfalfa (Miller et al., 1984) to *P. megasperma* Drechs; tobacco (Helgeson et al., 1976; Tedford et al., 1990) to *P. parasitica* Dast. var. *nicotianae* (Breda de Haan) Tucker; and apple (Barritt et al., 1990; Jeffers et al., 1981; Utkhede, 1986; Utkhede and Quamme, 1988) to *P. cactorum* (Leb. & Cohn) Schroet. Isolate specificity was noted in the reaction of papaya shoot cultures to *P. palmivora* (Butl.) Butl. (Sharma and Skidmore, 1988). Measurement of lesions formed on excised apple twigs after inoculation with *P. cactorum* has proven to be a good indicator of relative resistance (Barritt et al., 1990; Jeffers et al., 1981; Utkhede, 1986; Utkhede and Quamme, 1988). Preliminary investigations on the response of *Citrus* cultivars to culture filtrate from *P. citrophthora* were not particularly encouraging (Vardi et al., 1986a), although Tusa et al. (1988) obtained a fair correlation between in vitro tolerance to culture filtrate and in vivo tolerance to bark inoculations in sour orange selections. No investigations of in vitro response of *Citrus* cultivars in dual culture with *P. parasitica* have been reported.

CHAPTER 3

REDISCOVERY OF CARIBBEAN FORBIDDEN FRUIT AND EVALUATION OF ITS SIGNIFICANCE FOR CITRUS BREEDING

Introduction

A single introduction of grapefruit (*Citrus ×paradisi* Macfadyen) into Florida from the West Indies in 1823 has been identified in the literature as the original source of all known grapefruit germplasm. Essentially all grapefruit cultivars in the United States are bud sports or nucellar seedlings derived from this single introduction. On the basis of historical records as well as morphological and biochemical characteristics, the grapefruit is considered to have originated in the West Indies as one or a series of chance hybridizations between sweet orange (*C. sinensis*) and pummelo (*C. grandis*) during the 17th or 18th centuries (Scora et al., 1982; Scora and Kumamoto, 1983). In the first literature describing the grapefruit, it is closely associated with another kind of *Citrus* known as "Forbidden Fruit." Kumamoto et al. (1987) considered this form to be extinct. Subsequently, we identified a heterogeneous population of grapefruit-like *Citrus* growing in Saint Lucia (West Indies) that is known as forbidden fruit by some of the local residents (Bowman and Gmitter, 1990b). This population may provide a source of genetic diversity for grapefruit and hybrid cultivar development (Bowman and Gmitter, 1990a).

The objectives of this project were to:

1. Describe the *Citrus* populations known in Saint Lucia as forbidden fruit;

2. Investigate and describe the probable relationship of these populations to the forbidden fruit mentioned in the early Caribbean literature and to the grapefruit;

3. Survey the history of grapefruit and its connection with the Caribbean forbidden fruit;

4. Identify some of the needs of grapefruit genetic improvement and limitations imposed by currently available grapefruit germplasm;

5. Import (under quarantine) budwood and seeds of some of the Caribbean forbidden fruit selections for characterization and testing in Florida.

Materials and Methods

An extensive search for selections of the Caribbean forbidden fruit was initiated in Saint Lucia during 1986 and 1987. A travel grant was obtained (with F.G. Gmitter as co-investigator) from The Center For Tropical Agriculture/ International Programs (University of Florida) during 1987 to allow for the collection of seeds and budwood from the previously identified forbidden fruit selections. This trip was completed by F.G. Gmitter and K.D. Bowman during December, 1987, and included careful examination of five Saint Lucia forbidden fruit selections, collection of seeds and budwood, and discussions with Saint Lucians who were able to identify these trees as forbidden fruit. The budwood

selections were turned over to the Florida Department of Plant Industry, Gainesville, for shoot-tip budding, thermotherapy, and virus indexing. Additional seeds were obtained from one of these selections and another similar Saint Lucian selection in February and March, 1990. Seeds of morphologically similar selections were obtained from Saint Vincent and Trinidad in 1990.

Seeds of all selections were planted in soilless potting mix, and plants were grown in a greenhouse. Seeds of selections SF23, SF24, and SF25 were surface sterilized by immersion and agitation in 70% ethyl alcohol for 10 minutes followed by 1.05% sodium hypochlorite plus 2 drops polyoxyethylene-20-sorbitan monolaurate (Tween 20: Fisher Scientific, Pittsburgh) per 100 ml for 20 minutes. Seeds were rinsed five times (five minutes each) in sterile distilled water and placed on the surface of 10 ml agar-solidified half-strength MT basal medium with full strength iron (Murashige and Tucker, 1969) and 25 $g \cdot l^{-1}$ sucrose, adjusted to pH 5.7 (called GM1 medium through remainder of this text), that had been solidified in the bottom of 25 × 150 ml glass culture tubes (Bellco Glass, Inc., Vineland, NJ). The tubes were covered with "kap-uts" (Bellco Glass) translucent plastic closures.

Shoot tip cuttings (mericlones) from in vitro seedlings of selections SF23-1 and SF24-1 were excised and placed with the cut surface in RM1 rooting medium. RM1 medium was composed of agar-solidified half-strength MT basal medium with full-strength iron (Murashige and Tucker, 1969), 25 $g \cdot l^{-1}$ sucrose, 500 $mg \cdot l^{-1}$ activated charcoal, and 0.02 $mg \cdot l^{-1}$ NAA, and adjusted to pH 5.8, as described by Grosser et al. (1989). Epicotyl and internode segments (1 cm long) were excised from SF23-1 and SF24-1 and

placed horizontally on the surface of a callus proliferation media, composed of solidified MT basal (Murashige and Tucker, 1969) with 50 $g \cdot l^{-1}$ sucrose, 0.22 $mg \cdot l^{-1}$ kinetin, 0.5 $mg \cdot l^{-1}$ 2,4-D and adjusted to pH 5.8 (called CP1 media throughout this text), in 15 × 100 mm Petri plates.

After 6 weeks on callus proliferation medium, segments with associated callus were transferred to a shoot induction medium (SIM through the remainder of this text) composed of solidified MT basal (Murashige and Tucker, 1969) with 25 $g \cdot l^{-1}$ sucrose, 0.5 $g \cdot l^{-1}$ malt extract, 0.25 $g \cdot l^{-1}$ casein hydrolysate, 5 $mg \cdot l^{-1}$ BA, 0.01 $mg \cdot l^{-1}$ 2,4-D and adjusted to pH 5.8, in 20 × 100 mm Petri plates (SIM medium recipe, personal communication from J.W. Grosser). Adventitious shoots (somaclones) that formed on callused explants were excised and placed on rooting medium (RM1). Rooted mericlones and somaclones were transferred to soilless potting mix in controlled environment chambers. Mericlones were morphologically characterized with other control genotypes.

Leaf samples from imported budwood and seedling selections were used for isozyme analyses on horizontal gels containing 10% Connaught starch (Fisher Scientific) and sometimes supplemented with 0.15% electrophoresis grade agarose. Extracts from leaf samples were made by crushing small rectangles of chromatography paper (wicks) into the abaxial side of leaf tissue until they were green with cell juices immediately prior to gel loading (Torres et al., 1978). Isozymes were separated using a pH 5.7 histidine-citrate buffer (Cardy et al., 1981) or Tris-citrate buffer (Gmitter, 1985) with 3 hours electrophoresis at 50 ma constant current and 4°C. Activity stains used were PGM (phosphoglucomutase), PGI (phosphoglucose isomerase), PER (peroxidase), and PGD (6-phosphogluconate

dehydrogenase) on the histidine-citrate buffer system, or GOT (glutamate oxaloacetate transaminase), and SDH (shikimic acid dehydrogenase) on the tris-citrate buffer system. Stain recipes were as described by Vallejos (1983) with slight modifications (see also Almansa et al., 1989; Kephart, 1990; Normand, 1988).

Results and Discussion

The origin and history of contemporary grapefruit cultivars has been investigated through the literature (Figure 3.1). Essentially all the popular cultivars were derived from a single set of germplasm introduced to Florida about 1823. This germplasm and the grapefruit hybrid species itself probably originated someplace in the West Indies, where it was closely associated with another name or kind of *Citrus*, the forbidden fruit. Natural populations of grapefruit-like *Citrus* have not been evaluated as potential sources of germplasm. Initial observations of a diverse semi-wild population of *Citrus* called forbidden fruit in Saint Lucia, West Indies, indicated that it might be a useful source of genetic material and prompted more thorough evaluations and germplasm collections.

Forbidden fruit budwood is now growing under quarantine at the DPI facility in Gainesville and will be released after it is certified virus free. Seedlings of five selections are growing in the growth chamber, greenhouse, and/or field. Two manuscripts on the history, rediscovery, and characteristics of the forbidden fruit and its significance for grapefruit genetic improvement have been published (Bowman and Gmitter, 1990a, 1990b). Some of this published information is repeated here.

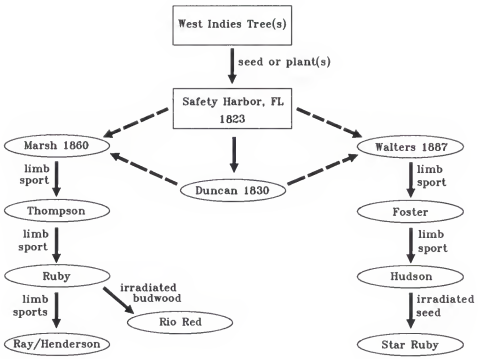

Figure 3.1. Probable pedigree of grapefruit cultivars (modified from Bowman and Gmitter, 1990a; p. 42).

Many Saint Lucians who were interviewed had never heard of forbidden fruit or only knew it was a kind of *Citrus* similar to grapefruit. However, two morphologically distinct kinds of *Citrus* were identified as forbidden fruit by some Saint Lucian citizens. The first of these morphologically resembled the species *Citrus grandis* and examination of seed samples indicated monoembryony. Only five trees of this kind could be located, although other trees were mentioned by residents. Two of these were known to have been vegetatively propagated by grafting from one of the other three trees and another was very unhealthy. The two healthy, mature (and probably seedling) trees were used as specimens for derivation of the following description. One of these trees was located on the

Raveneau Estate, Souci, Saint Lucia and identified as forbidden fruit by the resident estate owner, Fitz Raveneau. This tree was quite large and overgrown by vines and adjacent vegetation. Fruit sampled the first week of December, 1987, were very sour. Fitz Raveneau indicated that rind from the fruit of this tree was once used to make a type of candy. The second grandis-type tree used as a specimen to construct the description was growing on the Fond Doux Estate, Soufriere, Saint Lucia and was identified as forbidden fruit by Vernon, the resident estate manager. The original trunk of this tree was very large but oriented horizontally on the soil surface as a result of a hurricane in 1980. The "tree" that remained was composed of one large vertical branch that appeared to be healthy. Fruit of this tree were sweet and had a pleasant flavor when sampled between January-March, 1986 and 1987, as well as again in December, 1987. A composite description of these two trees from Bowman and Gmitter (1990b) is reproduced below.

Saint Lucia Forbidden Fruit, grandis-type (GF)

Trees to 12 m tall, trunk upright, branches spreading. Leaves evergreen, alternate; blade elliptic to ovate, 9-18 × 5-9 cm, glabrous above, glabrous with sparsely puberulent veins below, apex obtuse to acute, margins entire to crenate, base obtuse to cuneate, venation pinnate; petiole sub-alate to broadly alate, articulated, oblanceolate or obovate, occasionally with emarginate apex, 2-9 cm long. Young twigs green, angled, bearing single axillary spines, 0-25 mm long; mature twigs cylindrical, often spineless; older branches apparently spineless. Flowers not observed. Fruit borne singly or in pairs, a semi-sweet to acidic yellow hesperidium, oblate to pyriform, 20-25 cm × 15-20 cm; basal area slightly depressed, apex truncate; pedicel medium to very large; flavedo coarsely glandular and turning yellow at full maturity; albedo spongy, white, 1-4 cm thick; flesh greenish-yellow to pale white, 11-14 locules, with large, easily separated pulp-vesicles; central axis irregular, hollow. Seeds none to 100 per fruit, 15-20 mm × 6-12 mm, cream-color, obovoid, flattened, angular, ridged, rough, monoembryonic; inner seedcoat light brown, dark chalazal spot; cotyledons white. (Bowman and Gmitter, 1990b; p. 168)

A second kind of *Citrus* found growing sparsely throughout Saint Lucia was identified as the forbidden fruit of the past by MacDonald Ferdinand (born 1893) of Praslin, Micoud, Saint Lucia. Trees of this kind were somewhat smaller than the grandis-type and were more commonly known by Saint Lucians as "shaddette." Shaddette fruit varied considerably in characteristics from tree to tree, but were generally smaller and more similar to grapefruit or sweet orange than those of the grandis-type. Four shaddette trees were selected as specimens for the description reproduced from Bowman and Gmitter (1990b) below. Two of these trees were located near Fond-Saint-Jacques, Soufriere, one on the Fond Doux Estate, Soufriere, and the fourth in Blanchard, Micoud.

Saint Lucia Forbidden Fruit, shaddette-type (SF)

Trees to 10 m tall, trunk upright, branches spreading. Leaves evergreen, alternate; blade elliptic to ovate, 6-10 × 4-6 cm, venation pinnate, blade glabrous above and below, apex obtuse to acute or occasionally emarginate, margins entire to crenate, base obtuse; petiole sub-alate to alate, articulated, oblanceolate or obovate, sometimes with emarginate apex, 2-3 cm long. Young twigs green, angled, bearing single axillary spines, 2-5 mm; mature twigs cylindrical, sometimes spineless; older branches spineless or sometimes very stoutly spined. Flowers in small racemes or one to several in leaf axils, perfect, actinomorphic; calyx cup-shaped with 4-5 lobes; petals 4-5, white to cream; stamens many; stigma flattened globose. Fruit a semi-sweet hesperidium, oblate, globose, or pyriform, 9-15 × 9-12 cm, sometimes hanging in distinct clusters; basal area frequently with shoulder or neck, depressed, lobed; apex truncate to rounded; flavedo moderately glandular and turning yellow at maturity; albedo white to pinkish, 5-20 mm thick; flesh pale yellow to pale pink, 11-14 locules, medium to large semi-coherent pulp vesicles; central axis solid. Seeds 5-30 per fruit, 15-16 mm × 7-9 mm, white, angular-ovate, moderately rough, monoembryonic and polyembryonic; inner seedcoat light to medium brown, dark brown chalazal spot; cotyledons white.
(Bowman and Gmitter, 1990b; pp. 168-170)

Both types of forbidden fruit were sometimes known by a second name in the local French creole dialect. This name was spoken as though it

were spelled "fwee dayfwandee" in English and literally translates to "fruit forbidden." The name "forbidden fruit" was considered by Saint Lucians to have originated from the biblical story of the Garden of Eden.

The morphological descriptions of the field specimens indicated grandis-type forbidden fruit (GF) closely resembled selections of *Citrus grandis*, a monoembryonic species. Because of the monoembryony, all seedlings of *C. grandis* are genetically unique and some morphological variation among seedlings is expected. Consequently, the two plant sample used to construct the morphological description of GF above may be inadequate to encompass normal genetic variation within this kind of forbidden fruit.

Vegetative morphology of mature shaddette-type forbidden fruit (SF) trees in Saint Lucia was very similar to that of nearby grapefruit trees. However, as noted in the description above, fruit varied considerably from tree to tree, with some trees bearing more pummelo-like fruit, others grapefruit-like fruit, and yet others somewhat orange-like fruit. The four selections chosen as models for construction of the description above probably do not adequately sample all the variation in characteristics possible within the shaddettes. Examinations of small numbers of seeds from several selections indicated that at least some selections contained both monoembryonic and polyembryonic seeds.

In vitro seedling growth, callus induction on internodal explants, adventitious shoot formation, and shoot rooting were evaluated for one seedling selection of SF23 (SF23-1) and one seedling of SF24 (SF24-1). Both seedlings were observed to grow well in culture, produce large amounts of callus from explants, and form large numbers of adventitious

shoots from the callus. It is worth noting that adventitious shoot formation was much more prolific on explants from both shaddette selections than on explants from 'Valencia', 'Hamlin', and 'Ridge Pineapple' sweet oranges or 'Duncan' grapefruit. Excised shoots of both shaddette seedling selections were observed to root readily on RM1 medium. Samples of shoots from 'Carrizo', 'Hamlin', 'Valencia', and SF23-1 were examined for rooting at 20, 24, 28, and 32 days after placement on RM1 medium in culture boxes (Fig. 3.2). One month after placement on RM1, 33% of SF23-1 shoots, 27% of 'Carrizo' shoots, 18% of 'Hamlin' shoots, and 0% of 'Valencia' shoots had formed roots. After 44 days on RM1 medium, one of 23 'Valencia' shoots (4%) had formed a root.

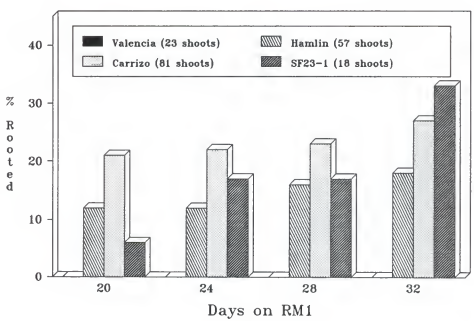

Figure 3.2. Frequency of shoot rooting during the first month on RM1 medium.

Meristem clones propagated by tissue culture were established in soilless potting mix and morphologically characterized (Tables A.1-A.6). Morphology of both shaddette clones differed considerably from 'Hamlin' and 'Valencia'. The shaddette seedlings closely resembled the 'Duncan' and 'Marsh' clones for the characteristics spine length, spine length/diameter, oil gland density, and internode length.

Isozyme analysis was completed on one of the two specimen GF clones (the other did not survive propagation in quarantine), as well one standard selection of sweet orange, grapefruit, and pummelo (previous work has indicated almost no variation within these species [F.G. Gmitter, personal communication; Torres et al., 1978]). Allelic constitutions of these clones were deduced from banding patterns of PGM, PGI, PER, SDH, and GOT activity stains (Table 3.1). Banding patterns of PGM, PGI, SDH, and GOT for samples from GF13 were identical with those observed for common Florida selections of pummelo, *C. grandis* (Table 3.1). Clone GF13 was observed to differ from common pummelo selections and resemble grapefruit in its heterozygosity (FS) at the peroxidase locus. These isozyme data are consistent with the possibility that GF13 is a hybrid between grapefruit (or shaddette) and pummelo, but not a simple hybrid between sweet orange and pummelo (because GF13 is homozygous FF at the GOT locus). However, a thorough survey of *C. grandis* has not been completed, and it is possible the F allele for peroxidase is present in some pure pummelo selections.

Isozymic characterization of the three shaddette clones (SF23, SF24, and SF25) was completed on plant material in quarantine. Allelic constitution of these clones and standard selections of sweet orange,

Table 3.1. Isozyme genotypes of seven *Citrus* selections.

	PGM	PGI	PER	SDH	GOT1
Sweet Orange	FS	MS	FF	IS	SS
Pummelo	SS	SS	SS	SS	FF, FS
Grapefruit	SS	SS	FS	IS	FS
SF23	SS	SS	FS	IS	FS
SF24	SS	SS	FS	IS	FS
SF25	SS	SS	FS	IS	FS
GF13	SS	SS	FS	SS	FF

SF = shaddette-type forbidden fruit
GF = grandis-type forbidden fruit

grapefruit, and pummelo were deduced from banding patterns of PGM, PGI, PER, SDH, and GOT activity stains (Table 3.1). The three shaddette clones and grapefruit had identical banding patterns for PGM, PGI, PER, SDH and GOT. Some preliminary evidence for differences in banding patterns of shaddette clones and grapefruit were obtained with PGD (personal communication, F.G. Gmitter). The shaddette selections were differentiated from pummelo by being heterozygous at PER (FS) and SDH (IS) loci. Shaddettes were distinguished from sweet orange by PGM, PGI, PER, and GOT. Allelic differences between the GF13 clone and the shaddette selections were detected with SDH and GOT as an increase in heterozygosity among the shaddettes.

Although the three shaddette clones were quite similar to grapefruit in morphologies and isozyme banding patterns, shaddette seedlings showed a much higher frequency of isozyme variation at the PER locus than seedlings from three grapefruit cultivars (Table 3.2; Gmitter et al., in preparation). Similar isozyme variation has been observed among shaddette seedlings at other isozyme loci (F.G. Gmitter, personal communication). The appearance of altered isozyme genotypes indicates that seedlings are zygotic in origin. An average 27 percent of shaddette seedlings showed recombination at the PER locus by the presence of FF or SS homozygotes. Because the seed parent clones are FS at peroxidase (Table 3.1), selfed zygotic progeny should segregate at a ratio of 1FF:2FS:1SS. Therefore, only about half of the zygotics would be detected as homozygotes at a single locus. Ignoring the possibility of cross pollination, the projected frequency of zygotics in a population of shaddette seedlings may be about 54 percent. This is in great contrast to the 0 to 8 percent zygotics predicted on the basis of the peroxidase segregation for the grapefruit cultivars.

Although the phylogenetic relationships cannot be determined precisely, isozyme evidence supports the morphological indications of a close relationship between the grapefruit and the Saint Lucian forbidden fruit. The connection between the two is further corroborated by the close association of the names in historical literature. Bowman and Gmitter (1990b) have proposed that the Saint Lucian shaddette and grapefruit should be considered as two different groups of selections within the hybrid species *C. ×paradisi*. The name "forbidden fruit" was apparently derived from an early *C. grandis* selection called "Adam's

Apple." The name forbidden fruit has subsequently been applied sporadically to many (all?) different selections of *C. grandis* and *C. ×paradisi* in the West Indian historical literature. The hypothesized species and nomenclatural relationships, as well as an indication of the genetic diversity within populations, are presented pictorially in Figure 3.3. Bowman and Gmitter (1990a) suggested that the shaddette may be a valuable germplasm resource, a proposal strengthened by the isozyme evidence presented here.

Table 3.2. PER zymotypes and percent zygotics identified among seedlings of grapefruit and three shaddette (SF) selections (Gmitter et al., in preparation).

	Number Seedlings Examined	PER Locus SS	FS	FF	Percent Zygotics Identified
Duncan	51	0	49	2	4%
Foster	50	0	50	0	0
Marsh	50	0	50	0	0
SF23	41	12	29	0	29
SF24	39	8	27	4	31
SF25	23	5	18	0	22

SF = shaddette-type forbidden fruit

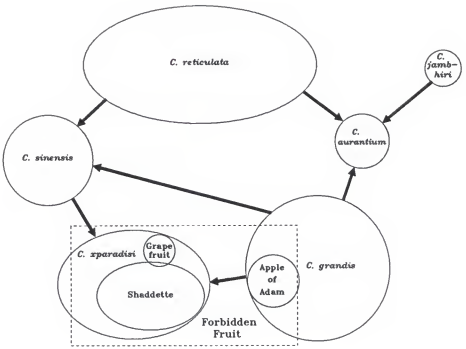

Figure 3.3. Speculative taxonomic and nomenclatural relationships between forms related to *C. ×paradisi* (modified from Scora, 1975; p. 373).

CHAPTER 4

EVALUATION OF ORGANOGENIC CITRUS TISSUE CULTURES AS A SOURCE OF GENETIC VARIATION FOR CULTIVAR IMPROVEMENT

Introduction

Numerous reports of variation among plants regenerated from tissue cultures have been recorded in the past two decades (D'Amato, 1985; Evans, 1989; Evans et al., 1984; Evans and Sharp, 1986; Orton, 1984). The term "somaclonal variation" was coined by Larkin and Scowcroft (1981) to describe this phenomenon. In some cases, useful mutations have been reported in somaclonal variants, including higher yield (Heinz and Mee, 1971), male sterility (Griesbach, 1989), disease resistance (Heinz et al., 1977; Krishnamurthi and Tlaskal, 1974; Orton, 1984; Shepard et al., 1980), insect resistance (Miles et al., 1981), dwarfing (Griesbach, 1989), and a wide variety of other morphological characteristics (Burk and Matzinger, 1976; Cummings et al., 1976; Eapen et al., 1989; George and Rao, 1983; Liu and Chen, 1978). The severe limitations on sexual hybridization of *Citrus* imposed by long juvenility and extensive nucellar polyembryony make somaclonal variation an attractive alternative method of generating useful genetic variation. Only a few preliminary investigations of somaclonal variation have been completed in *Citrus*, with somewhat conflicting results (Gmitter, 1985; Kobayashi, 1987; Navarro et al, 1985). No thorough

investigations of somaclonal variation arising from organogenic regeneration of *Citrus* cultures have been reported.

If somatic variation from *Citrus* tissue cultures is not frequent enough to make it productive in cultivar improvement, mutagenic treatments may be employed to increase genetic variation. Irradiation of budwood and nucellar seeds have both been successful in *Citrus* genetic improvement (Hearn, 1984, 1986; Hensz, 1977, 1985), and there have been some investigations of irradiation of *Citrus* tissue cultures (Chang et al., 1984; Kochba and Spiegel-Roy, 1982; Liu and Deng, 1985; Spiegel-Roy and Kochba, 1973b; Vardi, 1977; Zubrzycki and Diamante De Zubrzycki, 1982). However, little information is available on the effect of gamma radiation on *Citrus* organogenic and embryogenic callus cultures and the resulting plants.

This study investigated the potential for somaclonal variation in *Citrus* genetic improvement and studied the effects of gamma radiation on callus cultures. The somaclones were produced by organogenesis from internodal explants and compared with control plants produced by in vitro cuttings from apical and axillary meristems (mericlones) of the same plant material. The objectives were to:

1. Regenerate plants by organogenesis from callused *Citrus* explants in vitro;
2. Compare the juvenile morphology of these plants and plants produced by meristem cuttings from the same seedlings and/or from other seedlings of the same cultivar;
3. Determine the effect of gamma radiation on *Citrus* explants.

Materials and Methods

Production of somaclones. 'Hamlin' and 'Valencia' sweet oranges (*C. sinensis* [L.] Osbeck) were chosen for the study of somaclonal variation because they are common cultivars that might benefit by some types of somatic mutations. The cultivar 'Carrizo' citrange (*C. sinensis* × *P. trifoliata* [L.] Raf.), known to be responsive in culture, also was included because the two sweet orange cultivars did not produce shoots readily from callus during preliminary studies. Seeds of 'Carrizo', 'Hamlin', 'Valencia', 'Duncan' grapefruit (*C.* ×*paradisi* Macfadyen), shaddette selections SF23-1 and SF24-1 (presumably *C.* ×*paradisi*; see Chapter 3) and 'Meiwa' kumquat (*Fortunella crassifolia* Swing.) were surface sterilized by immersion and agitation in 70% ethyl alcohol for 10 minutes followed by a solution of 1.05% sodium hypochlorite plus two drops polyoxyethylene-20-sorbitan monolaurate (Tween 20: Fisher Scientific, Pittsburgh) per 100 ml for 20 minutes. The seeds were rinsed in sterile distilled water five times (five minutes each rinse) and individual seeds placed on the surface of 10 ml germination medium (GM1; see Chapter 3) solidified in the bottom of 25 × 150 mm glass culture tubes (Bellco Glass, Inc., Vineland, NJ). The tubes were covered with translucent plastic Kaputs closures (Bellco Glass) and sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan). In vitro seedling cultures were maintained at 27°C under constant fluorescent lighting.

After seeds germinated, 1 cm epicotyl and internode segments (called "segments" through the remainder of this chapter) were excised and placed horizontally on the surface of a callus proliferation medium, called CP1 (see Chapter 3). Some 'Carrizo' segments were sacrificed for

determination of the starting mass of apical and basal halves of the explants. 'Carrizo' segments that were to be used for the 21 day mass determinations were marked at their midpoint with filter-sterilized India ink prior to placement on CP1 medium. Severed seedling apices and/or nodes were retained as meristem clones (mericlones) and placed in rooting medium (RM1, see Chapter 3) in 20 × 100 mm Petri plates or Magenta GA-7 culture boxes (Magenta Corp., Chicago).

After 3 weeks on CP1, marked segments were removed for fresh and dry weight determinations. Segments that were to be maintained on CP1 medium for 6 weeks were transferred to fresh CP1 medium at three weeks. After 4 or 6 weeks on callus proliferation medium, explant segments with associated callus were transferred to a shoot induction medium, called SIM (see Chapter 3). Adventitious shoots (somaclones) that formed on callused explants were excised and placed on rooting medium (RM1) or minigrafted on etiolated 'Carrizo' seedlings (see below).

Rooted adventitious shoots and meristem clones were transferred to soilless potting mix and maintained in high humidity and moderate light for several weeks before being transferred to a controlled environment chamber where plants were grown prior to morphological evaluation. Similar methods were used to produce twelve 'Valencia' mericlones, five 'Valencia' somaclones, one minigrafted 'Duncan' somaclone, one 'Meiwa' mericlone, one shaddette SF23-1 mericlone, and three shaddette SF24-1 mericlones for use as genetically diverse controls (Table 4.1). One seedling from a ribbed fruit chimera of 'Marsh' grapefruit (*C. ×paradisi*) was used as another genetically different, unifoliolate control. Seven etiolated 'Carrizo' seedlings were decapitated between the cotyledons and

the first node and allowed to regrow by adventitious shoots as additional trifoliolate controls. All plants for this study were grown in 153 ml of a soilless potting mix (SPM2) containing peat moss, vermiculite, and perlite (Fafard Mix No. 2: Conrad Fafard, Inc., Springfield, MA) using pots 20.5 cm long and 4 cm inside diameter at the top.

Table 4.1. Clones used in somaclonal variation studies.

Cultivar	Clone type	Root stock	Number plants	Approximate number measurements per plant per characteristic
Carrizo	Seedling	Self	7	6
Carrizo	Mericlone	Self	4	6
Carrizo	Somaclone	Self	47	6
Duncan	Somaclone	Carrizo	1	5
Hamlin	Mericlone	Self	14	5
Hamlin	Somaclone	Self	29	5
Hamlin	Somaclone	Carrizo	8	5
Marsh	Seedling	Self	1	5
Meiwa	Mericlone	Self	1	3
SF23-1	Mericlone	Self	1	5
SF24-1	Mericlone	Self	3	6
Valencia	Mericlone	Self	12	5
Valencia	Somaclone	Self	3	5
Valencia	Somaclone	Carrizo	2	5

'Carrizo' seedlings used for minigrafting were grown in SPM2 and covered with tall unlighted tubes for maximum nonchlorophyllous shoot elongation and then decapitated by a horizontal cut between the cotyledons and first leaf node (1-5 cm above surface of SPM2). Shoot tips 1-3 mm in length were excised from the explant and immediately placed on the decapitated 'Carrizo' stock so that the cut surfaces of the two units were in contact (shoot tips were much larger than those used in previously-described micrografting [Nauer et al., 1983; Navarro et al., 1975; Oiyama and Okudai, 1986; Tusa et al., 1978]). The stems of the etiolated 'Carrizo' seedlings and the minigrafted adventitious shoots were of similar diameter.

After transfer to SPM2, all mericlones, somaclones, and minigrafted somaclones (also the 'Marsh' and 'Carrizo' seedlings) were grown in a controlled environment chamber with a diurnal cycle of 16 hr light at 30°C and 8 hr dark at 25°C. Plants were watered every two days, alternating between tap water and a solution of 20-10-20 fertilizer plus micronutrients at 300 $mg \cdot l^{-1}$ (Peter's Professional Peat Lite Special 20-10-20 Water Soluble: W.R. Grace & Co., Fogelsville, PA). Insects were controlled on three occasions by spray application of kinoprene (Enstar 5E: Zoecon Corp., Palo Alto, CA) plus oil or fluvalinate (Mavrik: Zoecon Corp.) within 10 days of a cutback.

Evaluation of plant morphology. Plants were cutback and allowed to regrow from axillary buds prior to the morphological evaluations so that the transient effects of tissue culture would be minimized and all organs would be the same age at the time of evaluation. Vegetative morphologies of 'Hamlin' mericlones and somaclones, 'Valencia' mericlones and

somaclones, one 'Meiwa' mericlone, one shaddette SF23-1 mericlone, three shaddette SF24-1 mericlones, one 'Duncan' somaclone, and one 'Marsh' seedling were evaluated 6-9 weeks after plants were cut back 10 cm above the soil level (or 10 cm above the graft union for the minigrafted 'Hamlin' somaclones and minigrafted 'Duncan' somaclone). Single shoots were allowed to grow on each plant and any additional axillary budbreak was removed. Characteristics measured during the first evaluation were leaf length, leaf width, internode length, spine length, spine diameter (at the spine midpoint), abaxial oil gland density, abaxial leaf color, and adaxial leaf color. Leaf, internode, and spine lengths, as well as leaf width, were measured with a steel ruler in millimeters. Spine diameter was measured by a dial caliper to the nearest thousandth of an inch and converted to the nearest tenth of a millimeter for calculations. Abaxial oil gland density was estimated by averaging counts from a microscope-video camera image of two 20 mm^2 fields per leaf. Abaxial and adaxial leaf colors were determined on the standard X, Y, and Z color axes by a HunterLab Citrus Colorimeter model D25 (HunterLab, Reston, VA) modified with a black-plastic faceplate containing a 19 mm circular aperture. Conversions of color measurements to the L, A, and B visual color axes were completed using standard mathematical formulae issued by HunterLab and similar to those described by Ting and Rouseff (1986). Morphological characterization, in most cases, was based on five nodes from each plant, beginning with the first node more than 3 cm above the start of the regrowth after the cutback and including each of the next 5 consecutive nodes. Organs obviously damaged or within 3 cm of a shoot apex were not included.

A second morphological evaluation of 'Hamlin' mericlones and selected somaclones was completed 14-16 weeks after a third shoot cutback. Single axillary shoots were allowed to grow on each plant, and any additional axillary budbreak was removed. Characteristics measured during the second evaluation were leaf length, leaf width, spine length, spine diameter (at the spine midpoint), abaxial leaf color, and adaxial leaf color. The second morphological characterization, in most cases, was based on five nodes from each plant, beginning with the first node more than 3 cm above the start of the second growth flush after the third cutback and including each of the next 5 consecutive nodes. Organs obviously damaged or within 3 cm of a shoot apex were not included.

Morphological evaluations of 'Carrizo' mericlones, somaclones, and adventitious seedlings were accomplished 20-22 weeks after the first cutback (for leaflet length and chirality) or 19-20 weeks after the second cutback (for spine length and diameter). Characteristics measured were chirality (bud to the left or right of the spine at each node), left, main, and right leaflet lengths, spine length, and spine diameter (at the spine midpoint). Spine L/D ratio and leaflet ratio (left plus right leaflet lengths divided by main leaflet length) were calculated. Characterization of leaflet length and chirality were based on six nodes from each plant, beginning with the first node more than 8 cm above the start of the regrowth after the first cutback and including each of the next six consecutive nodes. Characterization of spine length and diameter was based on six nodes from each plant, beginning with the first node more than 8 cm above the start of the second growth flush after the second

cutback and including each of the next six consecutive nodes. Organs obviously damaged or within 8 cm of a shoot apex were not included for any measurements.

Irradiation of embryogenic cultures. Irradiation of embryogenic callus cultures was investigated as a method of inducing a higher frequency of mutations among plants from tissue culture. All available embryogenic callus suspension lines were obtained from J.W. Grosser, including 'Hamlin', 'Valencia', 'Valencia Rohde Red' (Stewart et al., 1975), 'Ridge Pineapple' (*C. sinensis*), and 'Milam' (*C. jambhiri* Lush.). Suspension cultures 7 days old from each cultivar were plated out onto EME medium, composed of solidified MT basal (Murashige and Tucker, 1969) with 50 $g \cdot l^{-1}$ sucrose, 0.5 $g \cdot l^{-1}$ malt extract and adjusted to pH 5.7, in 15 or 20 × 100 mm Petri plates. After 18 days, plates were exposed to 0, 3, 6, 12, or 24 krad from a Cobalt-60 source. Callus or embryos were transferred to EME supplemented with 20 $ml \cdot l^{-1}$ coconut water about 12 weeks after irradiation. Germinating embryos were transferred to SPM2 and established in growth chambers.

Irradiation of organogenic cultures. Irradiation of organogenic explants or callus cultures was investigated as a method of inducing a higher frequency of mutations among plants from tissue culture. 'Carrizo' and 'Hamlin' were chosen for the same reasons described in the somaclonal variation project. In this study, 'Ridge Pineapple' was substituted for 'Valencia' because the latter had proved very difficult to regenerate by organogenesis. The hybrid US-119 ([*P. trifoliata* × *C.* ×*paradisi*] × *C. sinensis*) also was included as a trifoliolate type (and thus perhaps very responsive to culture) that might benefit by disruption of some metabolic

pathways affecting flavor components (one of the types of mutations likely to occur as a result of irradiation). US-119 is of interest as a cold hardy and relatively good-tasting scion type that could still benefit by the loss of some remaining trifoliolate flavor characteristics.

Seeds of 'Carrizo' citrange, 'Hamlin', 'Ridge Pineapple', and US-119 were surface sterilized by immersion in 70% ethyl alcohol for 10 minutes followed by a 1.05% solution of sodium hypochlorite (20% bleach) plus 2 drops Tween per 100 ml for 20 minutes. Disinfested seeds were then rinsed in sterile distilled water 5 times (5 minutes each rinse), and individual seeds were placed on the surface of 10 ml germination medium solidified in the bottom of 25 × 150 mm glass culture tubes covered with Kap-uts. All seedling cultures were maintained at 27°C under a diurnal cycle of 16 hr fluorescent light and 8 hr dark. After seeds germinated, 7 mm segments were excised from the stems and placed flat on the surface of CP1 medium in 20 × 100 mm Petri plates for callus induction. All callus cultures were maintained at 27°C and under continuous fluorescent lighting. Selected segments were weighed prior to irradiation and exposed to 0, 3, 6, 12, or 24 krad from a Cobalt-60 source six days or 41 days following explant placement on CP1 medium. After six weeks on CP1, explants and associated callus were transferred to SIM medium in 20 × 100 mm Petri plates. Combined callus and explants of material irradiated 6 days after explant placement were weighed 36-38 days after irradiation. Health of callus cultures was scored about 70 days after irradiation using a subjective five level rating scale for organogenic potential: 1 = dead, 2-4 = intermediate, and 5 = adventitious shoots present.

Results and Discussion

Subjective observation did not reveal differences between epicotyl and internodal segments in callus growth, adventitious shoot formation, or subsequent rooting of these shoots. However, substantial differences were evident in the responses of explants from the different cultivars. Explants from 'Carrizo' and the shaddette selections SF23-1 and SF24-1 produced callus more rapidly and shoots more profusely than explants of 'Hamlin' and 'Valencia'. Shoots of all these selections rooted to some degree in RM1 medium. 'Duncan' was distinguished by the production of large amounts of callus and adventitious shoots but an exceptional recalcitrance to rooting on RM1.

Callus growth from explants of all the cultivars examined exhibited considerable polarity, and usually the basal portion of each 1 cm segment produced substantially greater amounts of callus than the apical portion of the segment. An experiment was conducted to measure this differential callus growth. Fresh weight of 1 cm 'Carrizo' segments was found to be equally distributed between the apical 0.5 cm and basal 0.5 cm (N=12) when measured immediately following excision from the in vitro seedlings (Fig. 4.1). When maintained on CP1 medium, a much larger amount of callus growth occurred on the basal half of the explant as compared with the apical portion. The average fresh weight of the tissue that developed from the basal half of the explant was about five times greater than that from the apical half after 21 days on CP1 medium (N=8). Dry weight of these segment halves at the end of 21 days on CP1 averaged 19.4 mg for the basal section and 5.4 mg for the apical section. The polar growth response was similar regardless of the segment length or how many pieces

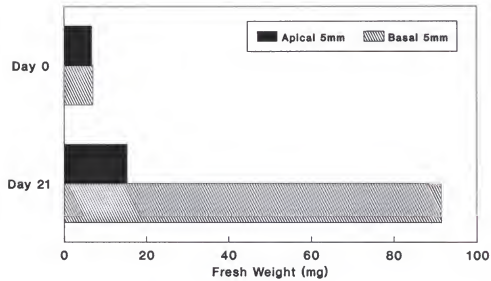

Figure 4.1. Differential callus growth in apical and basal portions of 1 cm 'Carrizo' segments on CP1 medium.

into which a single segment was divided. This effect may be the result of differential hormone (e.g. 2,4-D) uptake from the medium by the apical and basal ends of the explant. Although explants were oriented horizontally on the surface of the medium, there may have been a significant effect of xylem or phloem conductance polarity as determined during seedling development. Similar polarity of callus proliferation was noted in explants from 'Hamlin' and 'Valencia', although the total volume of callus produced and the differential was considerably less.

Shoot induction from callused explants was never observed on CP1 medium and typically did not occur until three or more weeks after transfer to SIM medium. This was probably a reflection of the need for

the hormone BA in the medium for shoot induction (Duran-Vila et al., 1989). Sporadic adventitious shoots were obtained from callused segments of 'Hamlin' and 'Valencia' on SIM medium, but most explants from these cultivars produced no adventitious shoots, even after 6-12 months on SIM medium. Although callused explants from 'Carrizo' and shaddette SF24-1 did not always produce adventitious shoots, they were more likely to produce shoots than 'Hamlin' or 'Valencia'. In addition, organogenesis from responsive individual explants of 'Carrizo' or SF24-1 was not sporadic, because scores of adventitious shoots could typically be obtained from the same explant. The 130 rooted 'Carrizo' somaclones obtained during this study were all produced by four explants from one nucellar 'Carrizo' seedling. These explants rapidly regenerated new shoots after each harvest and remained healthy and prolific until the experiment was terminated 12 months after first shoot induction. The nature of shoot induction on 'Carrizo' and shaddette explants suggests that each shoot may not be produced by an individual adventitious initiation event. Although initial shoot production must be regarded as adventitious because such shoots did not develop from preformed buds, the prolific nature of shoot induction from certain parts of the explant may result from the development of new meristematic regions in the callus material. Numerous buds and shoots may then develop from these meristems without additional adventitious events. If this is the case, the possibility for derivation of somaclonal variation from such regions is probably significantly less than it would be if each shoot developed from a separate adventitious initiation event. The formation of such an

"adventitious meristematic region" would, however, increase the potential of these techniques for micropropagation and/or genetic transformation.

Rooting of shoots on RM1 was easily achieved for 'Carrizo', shaddette SF23-1, and 'Hamlin', but was considerably slower for 'Valencia' (see Fig. 3.2). Different rooting media compositions were not evaluated for increased efficacy in this study, but the results of Duran-Vila et al. (1989) indicated that higher NAA concentrations may produce better results for unresponsive genotypes. A high percentage of the rooted shoots survived transfer to soilless potting mix (SPM2), especially if shoots and leaves were pruned prior to planting and high humidity was maintained in the acclimation chamber for several weeks following transfer. However, extending the period of high humidity or pruning roots prior to planting was distinctly detrimental to plant survival and establishment.

Minigrafting short adventitious shoots directly from the callus mass onto etiolated 'Carrizo' seedlings was investigated as a more rapid method of regenerating somaclones and one that might overcome selective forces eliminating certain types of mutants (e.g. mutations affecting rooting) from the regenerated plant population. Some success was achieved in the minigrafting of 'Hamlin' and 'Valencia' adventitious shoots, and ten of the forty-three somaclones included in the evaluated population were produced by this method. The one successfully regenerated 'Duncan' somaclone was also produced by minigrafting. These plants are indicated by a "Z" suffix in Tables 4.2 and A.1-A.17.

Many of the rooted adventitious shoots (somaclones) produced during this study exhibited deviant morphology during in vitro culture and the first few months of establishment in soilless potting mix. Elongated

leaves, extremely short internodes, and deformed shoots were observed among a number of the 'Hamlin' somaclones during the in vitro rooting and in vivo establishment phases. Similar aberrant morphologies, including unifoliolate leaves, were observed in some 'Carrizo' somaclones during these phases. To reduce the non-genetic (and ephemeral) alterations that would interfere with identification of true genetic mutations, morphological characterization was delayed until plants were well established in soil and was completed on healthy shoots grown entirely after a severe cutback in vivo. In addition, the principle control plants were produced from the same seedlings (or supposed genetically identical seedlings) by in vitro meristem cuttings (mericlones) that were rooted, established in potting mix, and grown under conditions identical to those used for the somaclones.

Morphological characteristics were chosen for use as indicators of genetic variation among the clones (mericlones and somaclones) on the basis of observed preliminary morphological abnormalities, convenience, and a presumed diversity of genetic controllers. In general, no practical techniques have been described for characterizing horticulturally and/or economically important traits in individual young *Citrus* plants (although evaluation of one such method was a goal of the research described in Chapter 6). However, if somaclonal variation is caused by random mutations, the frequency of mutations affecting several diverse morphological characters should indicate the mutation frequency affecting horticulturally important traits.

In the first morphological evaluation of the 'Hamlin' somaclones, fourteen 'Hamlin' mericlones were used as the reference population. These

mericlones were derived from ten seedlings that in turn were obtained from the fruit of one 'Hamlin' tree. These seedlings were expected to be of nucellar origin and genetically identical because 'Hamlin' is highly polyembryonic and has been observed only rarely to produce zygotic seedlings. Twenty-three of the 'Hamlin' somaclones were produced from these same seedlings or other seedlings obtained from the same tree at the same time. The remaining fourteen 'Hamlin' somaclones were produced from seedlings of a second 'Hamlin' tree considered to be morphologically indistinguishable from the first.

Normal probability plots (quantile-quantile) and the Kolmogorov test in the SAS univariate procedure indicated that the combined measurements of the fourteen 'Hamlin' mericlones (60 to 70 repetitions for each trait) were normally distributed for all of the evaluated characteristics (Prob.>D was greater than 0.01 for each character; Prob.>D was greater than 0.15 except for internode length, spine length, leaf length, and spine ratio). Therefore, these measurements were combined to obtain the "HAMLIN MERICLONE" used as the control in each of the statistical comparisons.

Student's t-tests were used to compare the morphological characteristics of the two subpopulations of 'Hamlin' somaclones that were obtained from different seed sources (indicated as "HA" and "HX" in the tables). Because all of the mericlones available for inclusion in the HAMLIN MERICLONE were from the "HA" population, any genetic differences between the two seed sources may have affected comparisons of the "HX" somaclones with the "HA" mericlones. The two populations had similar means (Prob.>t was at least 0.05) for all characters except leaf width

(Prob.>t = 0.0023), abaxial leaf color B (Prob.>t = 0.0383), and leaf L/W ratio (Prob.>t = 0.0003). Comparison of the variances for each characteristic in the two populations by the F statistic indicated that there was no evidence for differences between the variances of the two populations for most traits (H°: Variances are equal, Prob.>F was at least 0.1). Although some mean differences were indicated, no alternative control was available and the seed source was necessarily ignored in subsequent statistical comparisons. However, this factor must be considered as a possible uncontrolled source of variation that may have influenced the characteristics of individual "HX" somaclones or the resulting population statistics.

Student's t-tests were also used to compare the morphological characteristics of the 'Hamlin' somaclones on their own roots (29 clones) with the 'Hamlin' somaclones minigrafted on 'Carrizo' roostocks (8 clones). Differences in the effects of the two root types on morphological characteristics of somaclones would influence determinations of aberrant plants, and all of the mericlones included in the HAMLIN MERICLONE were on their own roots. Comparisons of these two subpopulations of 'Hamlin' somaclones by Student's t-tests indicated that the means were different (at $\alpha = 0.05$) for all of the morphological characteristics except internode length, leaf length, and leaf ratio. Root type significantly affected the morphological characteristics of the somaclones (e.g. 'Hamlin' on 'Carrizo' rootstocks tended to produce longer, thicker spines, and wider, less darkly colored leaves). However, this factor was ignored in subsequent statistical comparisons because no 'Hamlin' mericlones on 'Carrizo' rootstocks had been produced during the

generation phase of the study. The effect of rootstock must, therefore, be regarded as another uncontrolled source of variation in the statistical comparison of clone morphologies.

Twelve 'Valencia' mericlones (grouped as a single genotype with 50 to 60 repetitions), one 'Duncan' somaclone, one shaddette SF23-1 mericlone, three shaddette SF24-1 mericlones (grouped as a single genotype), one 'Marsh' seedling, and one 'Meiwa' mericlone were used in comparisons with the HAMLIN MERICLONE for each of the morphological traits. Duncan's mean separation ($\alpha = 0.01$) indicated that at least one clone was measurably different from 'Hamlin' in: adaxial leaf colors A, B, L, and A/B ratio; abaxial leaf colors B, L, and A/B ratio; spine length; spine diameter; spine length/diameter ratio; leaf width; leaf length/width ratio; oil gland density; and internode length (Tables 4.2 and A.1-A.6). The consistency with which these genotypes are clearly distinguished from 'Hamlin' gives an indication that genetic differences do, in fact, lead to measurable differences in many of these traits. However, it should be noted that the degree of genetic variation represented within this reference group was greater than would be expected to occur as a result of most somaclonal mutation. The cultivar 'Valencia' is most closely related to 'Hamlin' and is only separated from it by four characteristics with Duncan's test at $\alpha = 0.05$.

The first characterization of each of the 37 'Hamlin' somaclones and 5 'Valencia' somaclones is summarized in the first half of each column in Table 4.2. More complete results are presented in Tables A.7-A.12 where a mean measured value is indicated when it differed significantly from HAMLIN MERICLONE (Duncan's separation at $\alpha = 0.01$). Analysis of variance

probability of a greater F value is indicated at the end of each table column. More somaclones differed from HAMLIN MERICLONE than would be expected by chance for most of the morphological characters. For example, twelve of the forty-two somaclones differed from the mericlone in adaxial color L by Duncan's separation at $\alpha = 0.05$ (see Table A.7), while only 2-3 outliers are predicted by definition of the $\alpha = 0.05$ test. However, in general these aberrants were not distinctly different from all other somaclones but seemed to represent the tails of a broader normal distribution than that predicted by the mericlone controls. These observations suggest either an increased genetic diversity or some undefined nongenetic variation within the somaclone population.

In an attempt to determine whether the aberrations observed in these somaclones were caused by genetic mutations or nongenetic factors, the mericlones, the somaclones indicated to be aberrant during the first test, and some of the "normal" somaclones were cut back and regrown twice before a second morphological characterization. Measurements of oil gland density and internode length detected no variants during the first test (Table 4.2 and A.12.) and so were not included in the second characterization. Some mericlones and somaclones failed to regrow after the cutbacks and could not be reevaluated. For most of the characters, there were substantial differences between the mean values of the HAMLIN MERICLONE in the first and second morphological evaluations. This was probably caused by advanced maturity and the increased stress resulting from extended growth in relatively small rooting volumes. Nevertheless, it seems reasonable to assume that the resulting alterations were similar for each plant and should not have substantially affected relative

Table 4.2. Summary for morphological comparisons of HAMLIN MERICLONE with 6 other cultivars and 42 somaclones. See Tables A.1-A.17 for more complete data.

Clone	Adaxi Color A	B	L	R1	Abaxi Color A	B	L	R1	Spine LE	D	R2	Leaf LE	W	R3	In Lg	Oil Gl
Val M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Duncan	0	0	0	0	0	0	0	+	-	+	-	0	+	0	0	-
SF23-1	+	-	-	-	0	0	0	0	-	0	-	0	0	0	-	-
SF24-1	0	0	0	-	0	+	0	+	-	0	-	0	0	0	-	0
Marsh	-	+	+	0	0	+	+	+	-	0	-	0	0	0	0	0
Meiwa									-	0	-	0	-	+	-	+
HA142E121	+	-	-	0	+	-	-	0	0	0	0	0	0	0	0	0
HA142E215	00	00	00	00	00	00	00	00	0-	0-	--	00	00	00	0	0
HA142E222	0	0	0	0					0	0	0	0	-	0	0	0
HA4I221	0	0	0	0	0	0	0	0	0	-	0	0	-	0	0	0
HA5E121	0	0	0	-	0	0	0	0	0	-	-	0	0	0	0	0
HA5I122	0	0	0	0	0	0	0	0	-	-	-	0	0	+	0	0
HA6E114Z	0	+	+	0	0	0	+	0	0	0	0	0	0	0	0	0
HA7E12Z	0-	0+	0+	0+	00	0+	0+	0+	++	00	00	00	00	00	0	0
HA9E13Z	00	00	00	00	00	00	00	+0	+0	00	00	00	00	00	0	0
HX1X11	00	00	00	00	00	00	00	00	00	00	-0	00	00	00	0	0
HX1X12	00	00	-0	-0	00	-0	-0	-0	-0	-0	--	00	00	00	0	0
HX101E111	00	00	00	00	00	00	00	00	00	00	0+	00	00	-0	00	0
HX115E12Z	0-	0+	0+	0+	00	0+	0+	0+	00	00	00	00	00	00	0	0
HX115E14Z	00	00	00	00	00	00	+0	+0	00	00	00	00	00	00	0	0
HX15E11	0+	00	00	00	00	00	00	00	00	00	00	00	00	00	0	0
HX20E11	-0	+0	+0	00	00	+0	+0	00	00	00	00	00	00	00	0	0
VA13E11	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
VA30E11	0	0	0	0	0	0	0	0	0	0	0	0	0	+	0	0
VA33E11	0	0	0	0	0	0	0	+	0	0	0	0	0	+	0	0
VX91E11Z	0	+	+	0	0	0	+	0	0	0	0	0	0	0	0	0
VX91E12Z	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Other Hamlin Somaclones																
5 other	00	00	00	00	00	00	00	00	00	00	00	00	00	00	0	0
16 other	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

First and second columns in a category are for first and second test, respectively. HM = HAMLIN MERICLONE; Val M = Valencia mericlone; HA or HX = Hamlin somaclones; VA or VX = Valencia somaclones; SF = shaddette; Z = minigrafted on Carrizo; Adaxi = adaxial leaf surface; Abaxi = abaxial leaf surface; A, B, and L = those respective color axes as measured by HunterLab colorimeter; R1 = A/B; LE = length; D = diameter; R2 = spine LE/D; W = width; R3 = leaf LE/W; In Lg = internode length; Oil Gl = oil gland density. 0 = value not different from HM with $\alpha = 0.01$; + = value different and greater than HM with $\alpha = 0.01$; - = value different and less than HM with $\alpha = 0.01$.

comparisons. Several somaclones were distinctly separated from the MERICLONE by adaxial leaf color during the retest (Tables 4.2, A.13, and A.15). Significantly, the three somaclones with the greatest differences in the second evaluation were indicated to be normal for the same characteristics during the first evaluation (Tables 4.2 and A.7), and the two somaclones that were most clearly distinguished from the MERICLONE for these characteristics during the first test were indicated to be normal in the second morphological evaluation. No somaclones were found to be different from the HAMLIN MERICLONE for leaf length, leaf width, and leaf shape during the second test. Two somaclones differing from the MERICLONE for spine ratio during the first test, HA142E215 and HX1X12, differed similarly for this same characteristic during the second test (Table 4.2). One somaclone differing from the MERICLONE for abaxial leaf color ratio during the first test (HA7E12Z) differed similarly during the second test. These were the only three examples of confirmed morphological differences between 'Hamlin' somaclones and the HAMLIN MERICLONE. Six somaclones determined to be aberrant during the first morphological evaluation could not be reevaluated because of poor growth. It cannot be definitively determined whether the three somaclones that were confirmed morphologically aberrant were genetically different from 'Hamlin'. Growth to fruiting and/or extensive molecular characterization may produce more conclusive evidence. These results indicate that the frequency of detectable morphological mutants among 'Hamlin' somaclones produced by these techniques was less than 8-24% (3-9 of 37).

Forty-seven 'Carrizo' somaclones were evaluated for morphological characteristics and compared with the Z2 MERICLONE (two plants) derived

from the same seedling. Mericlones from two other 'Carrizo' seedlings (Z1 and Z3) and seven 'Carrizo' plants regenerated from decapitated seedlings were used as additional controls.

The left, main, or right leaflet lengths and leaflet ratios of the 'Carrizo' somaclones were not observed to differ significantly from those predicted by the Z2 MERICLONE. Only one to three of the 47 somaclones were significantly different from the Z2 MERICLONE at $\alpha = 0.05$, and one or none was different at $\alpha = 0.01$ (Tables 4.3, A.18, and A.20). It was concluded that there was no evidence for additional variation in leaflet lengths or leaflet ratios induced by the tissue culture process. In addition, it should be noted that one of the two other mericlones tested, the Z3 MERICLONE, was also separated from the Z2 MERICLONE (at $\alpha = 0.01$) by left and right leaflet lengths, indicating that morphological variation among seedlings may be at least as great as morphological variation among somaclones.

The number of 'Carrizo' somaclones with aberrant spine lengths and spine L/D ratios were similar to those with aberrant leaflet lengths and not different from those predicted by the Z2 MERICLONE distribution (Tables 4.3 and A.19). In contrast, 19 of 41 somaclones (46%) were separated from Z2 MERICLONE on the basis of spine diameter with $\alpha = 0.05$ (Table A.19). However, the significance of this finding is questionable because three of the six controls were also separated from the Z2 MERICLONE by Duncan's test on the basis of spine diameter. Perhaps the number of repetitions of the control Z2 MERICLONE (six from one plant) was insufficient to provide a good estimate of the morphological variability observed within the clone. Nonetheless, there was no evidence of a

Table 4.3. Summary for morphological comparisons of CARRIZO Z2 MERICLONE with 2 other mericlones, 7 seedlings and 47 somaclones. See Tables A.18-A.20 for complete results.

	Leaf				Spine			Ch
Clone	LL	ML	RL	R4	LE	D	R2	Re
Z1 mericlone	0	0	0	0	0	0	0	0
Z3 mericlone	+	0	+	0	0	+	0	0
Z11 seedling	0	0	0	0				0
Z12 seedling	0	0	0	0				0
Z13 seedling	0	0	0	0				0
Z14 seedling	0	0	0	0	0	+	0	0
Z15 seedling	0	0	0	0	0	0	0	0
Z17 seedling	0	0	0	0	0	0	0	0
Z19 seedling	0	0	0	0	0	0	0	0
Z2E1CB317	-	0	-	0				0
Z2E235	0	0	0	0	0	+	0	0
Z2E236	0	0	0	0	0	+	0	0
Z2E239	0	0	0	0	-	-	-	0
Z2E240	0	0	0	0	0	+	0	0
Z2I153	0	0	0	0	0	+	0	0
Z2I157	0	0	0	0	0	-	0	0
Z2I166	0	0	0	0	0	+	0	0
Z2I167	0	0	0	0	-	-	-	0
Other Z2 Somaclones								
33 other	0	0	0	0	0	0	0	0
5 other	0	0	0	0				0

MZ2 = Carrizo 2 Mericlone; Z2 = Carrizo 2 somaclone;
LL= left leaflet length; ML = main leaflet length;
RL = right leaflet length; R4 = (LL+RL)/ML; LE = length;
D = diameter; R2 = spine LE/D; Ch Re = chirality reversals.
0 = value not different from MZ2 with $\alpha = 0.01$;
+ = value different and greater than MZ2 with $\alpha = 0.01$;
- = value different and less than MZ2 with $\alpha = 0.01$.

greater frequency of morphological variants among the 'Carrizo' somaclones than among the population of 'Carrizo' seedlings.

The final morphological characteristic measured on the 'Carrizo' somaclones was chirality at the nodes (i.e., whether the bud was to the left or right of the spine). The actual chirality of a shoot was probably not an important indicator of genetic variation, because it is known that different seedlings of the same genotype, and even different growth flushes of the same plant, vary in their direction of spiral (Schneider, 1968; Schroeder, 1953). However, mericlones were never observed to possess nodes with different chirality in the same growth flush and observations of such chirality reversals in some somaclones were considered a possible indicator of morphological instability that was probably not linked with genetic alterations. Numbers of chirality reversals in six nodes (maximum reversals=5) were counted for the 'Carrizo' somaclones (Table A.20.) and compared to a table of binomial distributions. Only two (of 47) somaclones had more chirality reversals than that predicted at $\alpha = 0.05$ (greater than three reversals). The number of chirality reversals in the somaclonal population was not different from expected.

The morphological evaluations of 'Hamlin' and 'Carrizo' somaclones provided some evidence that more individual somaclones than expected were outside the normal distribution predicted by the mericlones. This may indicate the occurrence of mutations or a greater variance for some characteristics in populations of *Citrus* somaclones regenerated by organogenesis than in mericlone populations from the same explant material. An increase in the variability of quantitative traits has been

reported for somaclones of other species (Daub and Jenns, 1989; Larkin, 1985; Ozias-Akins et al., 1989).

When individual 'Hamlin' variants were retested after a substantial regrowth period, most of these somaclones were morphologically normal. These observations indicate that at least part of the enhanced variability in the somaclone populations was nongenetic variation of unknown origin. The 'Carrizo' somaclone population contained more aberrant plants than expected from the normal distribution predicted from the control mericlone for only one character (spine diameter). The seedling 'Carrizo' population demonstrated a similar frequency of deviation for the same characteristic. The frequency of morphological variants among 'Carrizo' somaclones may be no greater than the frequency of variants among nucellar seedlings.

The extensive morphological evaluation of these 89 somaclones did not provide evidence that the production of *Citrus* plants by the described organogenic procedures produced frequent or striking morphological variants or mutations. Minor genetic alterations may occur in individual *Citrus* somaclones and rare mutations may alter gross morphological features. One tetraploid from a confirmed diploid seedling was recovered in a 'Hamlin' somaclone population produced during subsequent studies and not included in the morphological analysis. Other *Citrus* plants regenerated from tissue culture have differed morphologically from the cultivar from which they were derived (J.W. Grosser, personal communication). However, the results of the present study indicated that such morphological variation in 'Hamlin' only occurred at a low frequency, and in 'Carrizo' did not occur at a frequency greater than that observed

in seedling populations. This evidence suggests that producing *Citrus* somaclones by organogenesis is not an efficient means of generating genetic variation that is obvious in juvenile plants. However, induced mutagenesis in vitro by gamma irradiation or other mutagens may increase the frequency of mutation in tissue culture material to a more useful level.

Preliminary experiments were conducted to evaluate the use of gamma radiation for mutagenesis of embryogenic and organogenic *Citrus* cultures. 'Ridge Pineapple', and 'Hamlin' embryogenic callus cultures produced normal embryos after exposure to 3, 6, or 12 krad of gamma radiation from a Cobalt-60 source. 'Milam' callus that had been irradiated with 24 krad produced green embryoid structures that did not develop normally nor regenerate plants. Plants from 'Hamlin' callus that received 3 and 6 krad, and plants from 'Ridge Pineapple' callus that received 12 krad appeared healthy and were transferred to soil. Morphological evaluations of these plants were not undertaken.

Gamma irradiation of segments greatly affected explant response of 'Ridge Pineapple', 'Hamlin', 'Carrizo', and US119. Initial fresh weights of 7 mm explants averaged 13 g for 'Ridge Pineapple' (N=14) and 15 g for US119 (N = 36). Average explant fresh weight of the four cultivars after 40-42 days on CP1 medium was 226 g (N = 32), while irradiation with 12 krad six days following transfer to the medium reduced the average explant weight of the four cultivars after 40-42 days to 67 g (N = 28) (Figure 4.2). Significant cultivar differences were observed (Table 4.4) with eight repetitions per treatment (except US119 at 24 krad, where N = 4). Callus growth from selection US119 was much more sensitive to gamma

irradiation than that from 'Carrizo'. Exposing callus that had been growing on CP1 medium for 46 days to gamma radiation affected the initiation of shoots by the callus after transfer to SIM medium. This is demonstrated by the organogenic ratings of callus 70 days post-irradiation and 65 days after transfer to SIM medium (Figure 4.3). A subjective rating scale was used to evaluate health of cultures: 1 = all callus dead, 2-4 = intermediate, and 5 = adventitious shoots present. Both methods of evaluation indicated that a dose of 3-5 krad would give about a 50% reduction in measured response. This radiation dose may be the most efficient to use for the production of mutants from organogenic *Citrus* cultures.

The results of this investigation did not indicate that regeneration by organogenesis from callused epicotyl or internodal explants resulted in substantial morphological variability among the resulting *Citrus* plants. A more definitive indication of the value of somaclonal variation for mutation breeding and the problems it might create for genetic engineering of *Citrus* must await fruiting of somaclone populations. The preliminary studies with embryogenic and organogenic cultures indicated that exposure to moderate levels of gamma radiation results in significant reduction in callus growth, organogenesis, and embryogenesis, but plants may still be regenerated. Gamma irradiation of *Citrus* callus cultures may be a valuable method of increasing the frequency of mutations.

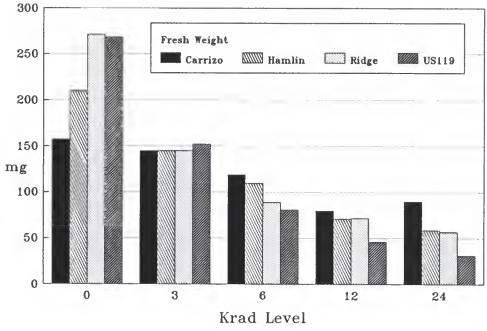

Figure 4.2. Effect of gamma radiation on callus fresh weight increase (N = 8 for all treatments except US119 at 24 krad, where N = 4).

Table 4.4. Fresh weight of callused explants 40-42 days post-irradiation. Cultivar means were separated using Duncan's multiple range test within krad levels (α = 0.05) as indicated by letters following values.

Krad	Carrizo	Hamlin	Ridge	US119	Mean	Prob.>F
0	157 b	210 b	271 a	268 a	226	0.0005
3	145	145	145	152	147	0.9855
6	119 a	110 ab	89 bc	81 c	100	0.0263
12	80 a	71 a	72 a	46 b	67	0.0319
24	90 a	59 b	57 b	31 c	63	0.0001

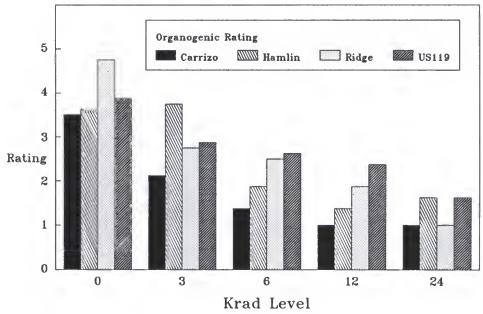

Figure 4.3. Effect of gamma radiation on organogenic rating: 1 = all callus dead, 2-4 = intermediate, and 5 = adventitious shoots present.

CHAPTER 5

CITRUS FRUIT SECTOR CHIMERAS AND THEIR POTENTIAL VALUE AS A GENETIC RESOURCE

Introduction

Many of the commercially important *Citrus* cultivars, such as 'Redblush' grapefruit (*C. ×paradisi* Macf.; probably the same as 'Ruby Red') and 'Rohde Red Valencia' sweet orange (*C. sinensis* [L.] Osbeck), arose as somatic mutations in previously existing selections (Hodgson, 1967; Mendel, 1981; Stewart et al., 1975). This fact, along with long juvenility and extensive nucellar polyembryony, has prompted increasing interest among *Citrus* breeders in utilizing natural and induced somatic mutations to obtain genetically superior cultivars (Hearn, 1986; Hensz, 1981; Russo et al., 1981).

Citrus fruit sector chimeras appear to be a type of somatic mutation (Cameron and Frost, 1968; Frost, 1943; Shamel, 1943; Toxopeus, 1933) but have received very little attention as a potential source of favorable genetic changes. Iwamasa et al. (1977) reported that the yellow-rinded sector of one chimeric fruit of the cultivar 'Fukuhara' produced seedlings that bore fruit with entirely yellow rinds. The normal orange-rinded sector of the same chimeric fruit produced seedlings that bore fruit with entirely orange rinds. Similar sectored fruit have been observed among many *Citrus* cultivars, and some of these bear mutant sectors that appear to have improvements in rind color or an increase in resistance to various

pests or diseases (personal communications, G.A. Moore and W. Grierson). Reports of an increased frequency for at least one type of sector mutation in response to treatment of flower buds with hydrogen cyanide (Sinclair and Lindgren, 1943) or chlorpyrifos (M.L. Arpaia, personal communication) suggest that the phenomenon may be manipulated by chemical applications to optimize the recovery of favorable mutations.

This study was initiated to investigate the potential for sectored *Citrus* fruit as a source of mutations to be used in genetic improvement. The objectives of this study were to:

1. Determine the approximate frequency and types of fruit sector mutations of some common *Citrus* cultivars by observations of graded fruit at commercial packinghouses;
2. Evaluate the potential for occurrence of desirable sector mutations;
3. Determine whether any plants regenerated from chimeric sectors were genetically abnormal.

Materials and Methods

Chimeric *Citrus* fruit were collected between November, 1988, and April, 1989, at two commercial fresh-fruit packinghouses near Lake Alfred, Florida. The cultivars sampled were 'Pineapple', 'Hamlin', and 'Valencia' sweet oranges (*C. sinensis*), 'Orlando' tangelo (*C. ×paradisi* × *C. reticulata* Blanco), 'Marsh' and 'Redblush' grapefruit (*C. ×paradisi*), 'Temple' (probably *C. reticulata* × *C. sinensis*) and 'Murcott' (probably *C. reticulata* × *C. sinensis*). Preliminary observations indicated that most sectored *Citrus* fruit were sorted into the number two grade and

eliminations categories by graders during normal packinghouse operations, and relatively few chimeras would be obtained from the number one grade or culls (see Soule and Grierson [1986] for a description of grade standards). Total numbers of fruit run through the packinghouse (all categories) during a sampling period were calculated on the basis of packinghouse records (number of field boxes run) and periodic sample counts (average number of fruit per box). To obtain an estimate of the frequency of sectored fruit among the different cultivars, all of the chimeric fruit were counted from the number two grade and eliminations categories while a known number of fruit was being run through the packinghouse. This method of sampling chimeric fruit was considered to yield a good estimate of the frequency and types of chimeras recovered from each cultivar sampled. For some cultivars, elimination and number two grade fruit were combined during packinghouse operations and only combined data were obtained. During the counting of chimeric fruit at the packinghouses, interesting or potentially useful fruit were collected and returned to the lab for more careful examination and/or plant regeneration.

Rind, flesh, and juice color were determined for normal and mutant sectors of selected chimeric fruit with a HunterLab Citrus Colorimeter model D25 (HunterLab, Reston, VA) using standard procedures (Redd et al., 1986). Rind puncture resistance was determined by an Instron model 1122 (Instron Corp., Canton, MA). Juice acidity and soluble solids were estimated by titration and refractometry, respectively (Redd et al., 1986).

Seeds extracted from mutant and normal sectors of some fruit were planted in soilless potting mix. When potentially useful chimeric sectors lacked viable seeds, undeveloped or aborted seeds were surface sterilized with a 1.05% solution of sodium hypochlorite and placed on EME medium (see Chapter 4). A small percentage of the undeveloped seeds from most sampled fruit eventually began to develop and produced viable embryos. Embryos that germinated by root and/or shoot elongation were subsequently transferred to soilless potting mix.

Seedling ploidy levels were determined by chromosome counts from root tips using a modification of the hematoxylin stain technique described by Sass (1958). The modified staining technique was developed by Professor X.X. Ling and is described below.

Young, white root tips were collected from plants growing in controlled environment chambers with a 16 hour photoperiod. Fresh root tips were washed in distilled water and placed in a saturated aqueous solution of 1,4-dichlorobenzene for 2-3 hours at room temperature. Then they were blotted dry and fixed in Carnoy's fluid (15 ml absolute [100%] ethyl alcohol: 5 ml glacial acetic acid) for 2-24 hours at room temperature. Following fixation, the root tips were hydrolyzed for 20 minutes in 5N HCl at room temperature and rinsed in distilled water. Then the tissues were transferred to a 4% solution of ferric ammonium sulfate for 2-4 hours at room temperature and thoroughly washed five times (five minutes each) in distilled water. Finally, tissues were stained in a 5% hematoxylin solution (0.5 g hematoxylin dissolved into 5 ml 95% ethanol, then 95 ml boiling water added to the hematoxylin-alcohol solution) for 2-4 hours and washed with distilled water twice. Samples were examined

by light microscopy at 400 to 1000 magnification after squashing onto a glass slide with 45% acetic acid. Chromosome number could be readily determined in cells undergoing active division.

Results and Discussion

Sectored chimeric fruit were defined as those fruit with one or more abnormal sectors and were readily distinguished from other types of mottled, scarred, or deformed fruit by straight margins diverging from the pedicel, widening near the equator of the fruit, and converging again to the stylar scar. Fruit with more than one distinct abnormal sector on the same fruit were not uncommon and were counted as a single chimera. These multiple chimeric fruit were classified according to the predominant sector change. Frequently, multiple chimeric sectors on the same fruit were either similar to each other in type or were opposite in effect (e.g. dark-red and light yellow sectors on normal pink grapefruit; see discussion below).

Substantial differences were observed in the frequency of chimeras among the eight cultivars that were examined (Table 5.1.). Chimeras were more frequent among the sweet orange cultivars 'Valencia', 'Pineapple', and 'Hamlin' than in the two grapefruit cultivars 'Redblush' and 'Marsh'. The mandarin hybrids were the most variable group, producing the highest ('Orlando' 0.271%), the mean ('Temple' 0.11%), and the lowest ('Murcott' 0.009%) frequencies of fruit sector chimeras. Repeated samples were not available for statistical comparison of frequencies for the different cultivars. However, three samples of eliminations 'Hamlin' fruit from one block and a fourth sample from a second block were examined in the

Table 5.1. Number and frequency of fruit sector chimeras from eight cultivars.

Cultivar	Sample size $\times 10^3$	Number fruit with chimeric sectors	Percent chimeric fruit
Orlando	139	377	0.271
Valencia	337	664	0.197
Pineapple	430	720	0.167
Temple	181	200	0.110
Hamlin	399	327	0.082
Redblush	161	66	0.041
Marsh	117	20	0.017
Murcott	519	46	0.009

packinghouse. Numbers of chimeric fruit in the three samples from the first block were 145 in 399,000 (total fruit run), 142 in 360,000, and 98 in 220,000, while the sample from the second block yielded 82 chimeras from a sample of 211,000 total fruit run. These represented frequencies of 0.036%, 0.039%, 0.045%, and 0.039%, respectively. The great similarity in frequency among the samples from the same cultivar suggests the possibility that chimera frequency may be a cultivar characteristic.

Comparison of chimeras counted in number two grade (Table 5.2) with those observed in the eliminations (Table 5.3) indicated that the latter category contained more of the mutant fruit. However, both groups yielded large numbers of chimeras of all types and would probably be equally valuable in the search for useful mutations. Collection of sectored fruit

Table 5.2. Number of fruit sector chimeras of different types in number two grade.

Cultivar	Sample size × 10^3	Number fruit with chimeric sectors					
		Gigas[z]	Sunken	Red/ Orange	Green	Black/ Brown	Other
Orlando	139	46	1	0	1	34	86
Pineapple	430	39	45	5	11	71	102
Hamlin	399	57	17	0	12	90	6
Redblush	161	4	1	3	4	16	7
Murcott	519	1	1	0	3	15	0

[z] Gigas = sectors with raised, thickened rind.

Table 5.3. Number of fruit sector chimeras of different types in eliminations.

Cultivar	Sample size × 10^3	Number fruit with chimeric sectors					
		Gigas[z]	Sunken	Red/ Orange	Green	Black/ Brown	Other
Orlando	139	16	2	1	0	46	144
Pineapple	430	69	73	7	5	120	173
Hamlin	399	54	6	0	4	76	5
Redblush	161	4	5	3	3	12	4
Murcott	519	1	1	0	0	17	7

[z] Gigas = sectors with raised, thickened rind.

from grading lines at commercial packinghouses was noted to be a much more efficient way to obtain large numbers of sector chimeras than searching among fruit on the trees. Arrangements could probably be made with cooperative packinghouse managers for instructing graders to collect particular types of sectored fruit. This would make it possible for the researcher to recover even extremely rare mutations with a minimum of labor.

Many different types of sector changes were observed in chimeric *Citrus* fruit. Sectors having alterations in rind color were most common and included black, brown, green, yellow, white, darker-red, and darker-orange. Numbers of fruit in some of these categories are indicated in Tables 5.2 to 5.4. Darker-orange or darker-red sectors were particularly

Table 5.4. Number of fruit sector chimeras of different types in combined eliminations and number two grade.

Cultivar	Sample size $\times 10^3$	Number fruit with chimeric sectors					
		Gigas[z]	Sunken	Red/ Orange	Green	Black/ Brown	Other
Valencia	337	102	86	19	61	189	207
Temple	181	21	35	6	34	74	30
Marsh	117	6	0	0	0	6	8

[z] Gigas = sectors with raised, thickened rind.

interesting because these characteristics would be favorable changes in many sweet orange and grapefruit cultivars. These darker pigmented sectors were observed among the fruit of 'Orlando', 'Pineapple', 'Redblush', 'Valencia', and 'Temple' (Tables 5.2 to 5.4). Some 'Hamlin' fruit with dark-orange sectors were recovered during a later study not reported in this text. One dark-red sectored 'Redblush' grapefruit was examined by several laboratory methods (Table 5.5). This particular chimera had both darker-red and lighter-yellow sectors on the otherwise normal faint-pink rind of the fruit. The rind of the mutant dark-red sector was much more highly pigmented than the normal part of the fruit, as indicated by the nearly 6-fold increase in the A/B ratio measured by a HunterLab Colorimeter. Flesh color of this chimeric sector was darker-red as well; this was documented by the near doubling of the flesh A/B ratio and the Citrus Redness (Ting and Rouseff, 1986) parameter of the juice color. Rind of the dark-red sector was also substantially more resistant to puncture than the normal part of the fruit. Other characteristics of the dark-red sector (soluble solids, acid, and brix/acid ratio) were not significantly different from the normal part of the fruit. The light-yellow sector of this fruit was too narrow to allow measurement of any parameters other than puncture resistance. However, it is interesting to note that this sector was less resistant to puncture than the normal part of the fruit, a change opposite to that observed in the adjacent dark-red sector. This may be additional support for the hypothesis that the mechanism of mutation in this case was somatic segregation (see discussion below).

Table 5.5. Summary of internal and external characteristics of mutant and normal sectors of 'Redblush' chimera 11.

	Normal sector	Dark-red sector	Yellow sector
Rind color			
A/B ratio	0.13	0.72	nd
(s.d.)	(0.06)	(0.04)	
Flesh color			
A/B ratio	0.77	1.25	nd
(s.d.)	(0.08)	(0.14)	
Juice color			
CR	17.7	31.5	nd
CY	31.4	36.8	nd
N	28.3	31.5	nd
Soluble solids			
°brix	7.9	8.1	nd
(s.d.)	(0.12)		
Acid	1.15	1.08	nd
(s.d.)	(0.13)		
TCB/acid ratio	7.0	7.7	nd
Puncture resistance	2.4	3.3	1.9
(s.d.)	(0.3)	(0.3)	(0.4)

s.d. = standard deviation; nd = no data; A/B = color ratio (Ting and Rouseff, 1986); CR = citrus redness by Hunter colorimeter (Ting and Rouseff, 1986); CY = citrus yellow by Hunter colorimeter (Ting and Rouseff, 1986); N = color score (Redd et al., 1986); TCB = total corrected brix (Redd et al., 1986).

Green sectored fruit were another type of color chimera that was observed among all cultivars except 'Marsh'. Green sectors observed in packinghouse fruit may be caused by resistance to degreening, or a lack of carotenoid or lycopene pigment development. In general, these would be unfavorable mutations. However, green sectors may also signify changes in the fruit maturation process. There could be some benefits in the development of clones that matured later but were otherwise identical to existing selections. One chimeric fruit with a very wide green sector was discovered on a navel orange tree in the field. This sector could be compared with normal fruit from the same tree as well as with the normal part of the chimeric fruit (Table 5.6). Navel orange is a cultivar group in which selections with different maturity dates have proven very valuable in the Australia (Tolley, 1989) and California citrus industries (Pehrson and Ivans, 1988). The A/B ratio of the rind from the green sector was measurably different from the normal part of the chimeric fruit (Table 5.6), while the rind color of the normal sector was essentially the same as that of the normal fruit from the same tree. The A/B ratio of the flesh in the green sector was less than that of the normal part of the chimeric fruit, but the differences were not as great as in the rind. Other characteristics of the green sector indicated less maturity (less juice color, more acid, lower soluble solids) than normal fruit from the same tree, but in general the internal characteristics of the normal sector of this particular fruit more closely resembled the green sector than it resembled the normal fruit from the same tree. One possible explanation for this observation is that the different flesh sections of a *Citrus* fruit may be associated in the expression of the characteristics

Table 5.6. Summary of internal and external characteristics of mutant and normal sectors of navel chimera 11.

	Navel chimera 11		
	Normal sector	Green sector	Normal fruit from same tree
Rind color			
A/B ratio	0.41	-0.07	0.43
(s.d.)	(0.13)	(0.18)	(0.03)
Flesh color			
A/B ratio	0.15	0.08	0.20
(s.d.)	(0.07)	(0.04)	(0.03)
Juice color			
CR	29.8	26.5	35.4
CY	68.7	64.6	73.4
N	35.0	34.0	36.4
Soluble solids			
°brix	10.8	10.6	11.5
(s.d.)		(0.14)	(1.25)
Acid	0.95	0.92	0.58
(s.d.)		(0.04)	(0.10)
TCB/acid ratio	11.6	11.7	20.0
Puncture resistance	1.8	2.3	1.9
(s.d.)	(0.1)	(0.2)	(0.2)

s.d. = standard deviation; A/B = color ratio (Ting and Rouseff, 1986); CR = citrus redness by Hunter colorimeter (Ting and Rouseff, 1986); CY = citrus yellow by Hunter colorimeter (Ting and Rouseff, 1986); N = color score (Redd et al., 1986); TCB = total corrected brix (Redd et al., 1986).

of maturity, and a mutation expressed in 75% of the flesh tissues may effectively alter the maturity of the entire fruit. Alternatively, the mutation may not be restricted to the flesh tissues beneath the mutant rind sector and may be present throughout the flesh.

The most common color changes observed in sector chimeras were to black or brown (Tables 5.2 to 5.4). Many of these chimera mutations appeared to result in increased rind susceptibility to one or more pests or diseases. This study did not recover any sectored fruit where the mutation appeared to result in increased pest resistance. However, chimeric sectors with resistance to melanose (*Diaporthe citri* [Fawc.] Wolf) and rust mite (*Phyllocoptruta oleivora* Ashmead) have been observed by other workers (W. Grierson, personal communication) and may be an important genetic resource.

Many fruit sector chimeras were not observed as changes in color, but rather as changes in rind texture or thickness. The two most common of these mutations (Figures 5.2 to 5.4) led to increased rind thickness (termed "gigas") and decreased rind thickness (termed "sunken"). Seedlings recovered from gigas sectors of three 'Valencia' and one 'Orlando' chimeric fruit were tetraploid (2n=4x=36). No seedlings were obtained from normal sectors of these same chimeric fruit. However, seedlings obtained from normal sectors of other gigas chimeric fruit were diploid (2n=2x=18). Because the cultivars 'Valencia' and 'Orlando' are polyembryonic and seedlings are normally of nucellar origin, these observations suggest that gigas chimeras are, in fact, cytochimeras with the gigas sectors composed of tetraploid cells. Tetraploids obtained from mutations in nucellar tissue of polyembryonic cultivars are expected to

be the result of somatic doubling (autotetraploids), and this has been confirmed by identical isozyme banding patterns for the tetraploids and their counterpart diploid cultivars (data not shown). The generation of tetraploid seedlings from one type of sector chimera has provided evidence that mutations expressed in the rind of sectored fruit may be recovered in seedlings.

Seedlings recovered from gigas sectors of monoembryonic cultivars (e.g. 'Temple') should be of zygotic origin. These zygotes should result from the fusion of a diploid (unreduced, 1n=2x=18) egg and either pollen from the same flower sector (unreduced, 1n=2x=18) or pollen from elsewhere (reduced, 1n=1x=9 or unreduced, 1n=2x=18). It will be interesting to see whether plants from gigas sectors of monoembryonic cultivars are diploid (unfertilized diploid egg or haploid egg plus haploid pollen), triploid (diploid egg + haploid pollen), allotetraploid (diploid egg + diploid foreign pollen), or autotetraploid (diploid egg + diploid self pollen). Triploid selections may be valuable as seedless cultivars, and tetraploid selections of existing cultivars may be useful as parents for the production of other seedless triploid hybrids (Soost and Cameron, 1968, 1980).

Sunken sectors were often observed adjacent to gigas sectors on chimeric fruit, suggesting the possibility of an origin by somatic segregation. If this were the case, such sectors could be haploid (2n=1x=9) or aneuploid. Viable haploid or aneuploid *Citrus* plants would be of interest for some types of breeding and genetic studies.

The origin of some fruit sector chimeras may be somatic segregation because occasionally two adjacent, contrasting sectors (twin sectors) were

observed, such as dark-red and light-yellow sectors on normal slightly-pink 'Redblush' grapefruit rind (Table 5.5), or the gigas-sunken twin sectors. In other cases, sector chimeras appeared to result from spontaneous polyploidization, as with the gigas chimeric fruit (without twin sunken sectors) from which tetraploid seedlings were recovered. It is also likely that some sector chimeras were the result of point mutations, transposable element activity, chromosome rearrangements, or other genetic mutations.

Most reports of *Citrus* fruit sector chimeras suggested that they are sectorial, implying that all cells within the affected sector are of the mutant type (Cameron and Frost, 1968; Iwamasa et al., 1977). It is likely that at least some fruit sector chimeras are mericlinal, with only the cell layer producing the rind being affected. The lack of darker flesh color in some dark-rinded chimeric fruit may be caused by the restriction of the mutation to the L-II layer; the flesh in these mericlinal chimeric fruit remains genetically and colorimetrically normal because it is derived from L-I tissue (Soost and Cameron, 1975). This effect is similar to the differential pigmentation in these tissues of the periclinal grapefruit cultivars 'Thompson', 'Foster', and Burgundy' (Soost and Cameron, 1975). Fortunately, *Citrus* nucellar embryos are derived from the L-II layer, as is the fruit rind (Soost and Cameron, 1975). Therefore, rind mutations that are mericlinal chimeras should be recovered in all three histogenic layers in seedlings. Seedlings grown from chimeric fruit are expected to be non-chimeric, whether normal or mutant, because zygotic and nucellar *Citrus* embryos originate from single cells (Bacchi, 1943; Osawa, 1912).

Width of mutant sectors varied from less than one millimeter to almost the entire fruit. Wider sectors may indicate that the mutation occurred earlier during fruit ontogeny and thus is more likely to be found in the seed. However, sector width probably also is affected by the positive or negative influence of the mutation on cellular metabolism and tissue physiology. Boundaries of chimeric rind sectors aligned perfectly with internal fruit septa in some cases, but more often there was no alignment. It is unclear whether this characteristic has any bearing on type of chimera (mericlinal or sectorial) or likelihood of mutant recovery. Tetraploid seedlings were recovered from both aligned and unaligned gigas chimeras.

The value of gigas fruit sector chimeras as a source of autotetraploid seedlings from commercial polyembryonic *Citrus* cultivars was demonstrated in this study. The reports of increased gigas sector formation following hydrogen cyanide fumigation (Sinclair and Lindgren, 1943) or chlorpyrifos application (M.L. Arpaia, personal communication) suggest that this phenomenon may be manipulated to maximize polyploid recovery. Tetraploids are valuable in breeding triploid seedless hybrids (Soost and Cameron, 1968, 1980). Observation of several other types of sector mutants that appear to be superior to the normal phenotype points out the potential usefulness of this resource. Mutants with darker fruit rind coloration would greatly enhance visual appeal of fresh fruit from many *Citrus* cultivars, thus reducing the need for ethylene degreening (McCornack and Wardowski, 1977) and application of dye (Kaplan, 1986) for consumer acceptance. Selections with increased flesh and juice color or altered maturity date could significantly increase grower income and

improve marketability of Florida and California citrus. Finally, reported observations of pest and disease resistant sector chimeras suggest that this genetic resource may have tremendous potential for the production of selections that yield high quality fruit with minimal pesticide application.

CHAPTER 6

RESPONSE OF STEMS FROM IN VITRO-GROWN SEEDLINGS TO PHYTOPHTHORA PARASITICA IN DUAL CULTURES

Introduction

Phytophthora parasitica Dast. is a soil-borne pathogen of *Citrus* in most tropical and subtropical regions of the world. *P. parasitica* infection frequently results in extensive necrosis of the feeder roots of susceptible *Citrus* rootstocks (root rot) and may also cause extensive damage to the cambial tissue under the bark, called foot rot or gummosis (Timmer and Menge, 1988). Clones with resistance/tolerance to both diseases caused by *Phytophthora* are available within *Citrus* and among related genera (Carpenter and Furr, 1962; Grimm and Hutchison, 1977), but each of these selections has other undesirable attributes that limit their usefulness as rootstocks in commercial orchards (Castle, 1987; Castle et al, 1989). Although chemical control of the diseases is possible (Davis, 1981; Menge, 1986; Sandler et al., 1989; Timmer, 1985), a breeding program for *Citrus* should ideally include at least moderate resistance or tolerance to the *Phytophthora* diseases as one of the desirable attributes of any newly developed rootstock. Unfortunately, resistance to *P. parasitica* is probably controlled by a multiple gene system (Hutchison, 1985), and no rapid, simple test for accurate determination of *Citrus* susceptibility to the pathogen is known. Although several different tests of *Phytophthora* resistance/tolerance have been described for *Citrus*

(Cameron et al., 1972; Carpenter and Furr, 1962; Graham, in press; Grimm and Hutchison, 1973; Smith et al., 1987; Tsao and Garber, 1960; Whiteside, 1974), these procedures are very time-consuming and results do not always agree with observed field susceptibility. Observations of seasonal fluctuations in plant susceptibility (Matheron and Matejka, 1989) further complicate interpretation of short-term field or greenhouse testing.

Some success has been achieved in characterizing or selecting for plant resistance to diseases in vitro (Behnke, 1979, 1980; Buiatti and Scala, 1985; Wenzel et al., 1985). In vitro resistance of plant tissues to *Phytophthora* in dual cultures has been found to correlate with field performance for several crop species (Barritt et al., 1990; Dolan and Coffey, 1986; Helgeson et al., 1976; Ingram, 1967; Jeffers et al., 1981; McComb et al., 1987; Miller et al., 1984; Tedford et al., 1990; Utkhede and Quamme, 1988; Warren and Routley, 1970; Zilberstein and Pinkas, 1987). Investigations on the response of *Citrus* cultivars to culture filtrate from *P. citrophthora* were not particularly encouraging (Vardi et al., 1986). However, no investigations of in vitro response of *Citrus* cultivars in dual culture with *P. parasitica* have been reported.

This project was initiated to investigate the possibility of using the in vitro reaction of *Citrus* seedling shoots to the pathogen as an indicator of field susceptibility to *Phytophthora* or as a technique for the study of resistance mechanisms under carefully controlled environmental conditions. The test conditions were not intended to imitate the response to either disease (root rot or foot rot) induced by *P. parasitica* on *Citrus*, but rather to examine general tissue response to the pathogen. The specific objectives of the study were to:

1. Determine whether *P. parasitica* can invade excised shoots of *Citrus* seedlings in dual cultures;
2. Determine the response of seedling shoots from several *Citrus* selections to *P. parasitica* in vitro;
3. Compare these responses with the accepted field susceptibilities of mature trees.

Materials and Methods

Pure isolates of *Phytophthora parasitica* that had been recovered from *Citrus* orchards near Ft. Pierce, Florida (called *P. parasitica* 'Riverland') and Bartow, Florida (called *P. parasitica* 'Hall') were obtained from J.H. Graham. These pure *P. parasitica* cultures were maintained on corn meal agar (BBL Microbiology Systems, Becton Dickinson and Co., Cockeysville, MD) or as in vitro stem infections on *Citrus* shoots. Rate of *P. parasitica* 'Hall' growth on corn meal agar at 27°C in continual darkness was estimated by measurement of distance from point of inoculation to culture margin at 2, 3, 4, 5, and 6 days after inoculation.

Plant tissue culture medium for dual culture tests was inoculated with agar plugs from the actively growing margin of pure *P. parasitica* colonies on corn meal agar or by segments of in vitro-infected seedling shoots from other dual cultures. The completely defined plant tissue culture media RM1 and GM1 (see Chapter 3) supported the growth of a luxuriant surface lawn of *P. parasitica* during these tests.

Experiments 1-3. Seeds of 'Valencia' and 'Hamlin' sweet oranges (*Citrus sinensis* [L.] Osbeck), 'Carrizo' citrange (*C. sinensis* × *Poncirus trifoliata* [L.] Raf.) and 'Swingle' citrumelo (*P. trifoliata* × *C.*

×*paradisi* Macf.) were surface sterilized by immersion and agitation in 70% ethyl alcohol for 10 minutes followed by 20% bleach (1.05% sodium hypochlorite plus two drops polyoxyethylene-20-sorbitan monolaurate [Tween 20: Fisher Scientific, Pittsburgh] per 100 ml) for 20 minutes. The seeds were rinsed in sterile distilled water five times (five minutes each rinse) and individual seeds placed on the surface of 10 ml GM1 medium (see Chapter 3) solidified in the bottom of 25 × 150 mm glass culture tubes (Bellco Glass, Inc., Vineland, NJ). The tubes were covered with translucent plastic Kap-uts closures (Bellco Glass, Inc.). Seedling and dual cultures were maintained at 27°C under constant fluorescent lighting.

At about three months (Experiment 1) or two weeks (Experiment 2) after seed germination, shoots were excised above the cotyledons and the base inserted several millimeters into solidified RM1 medium in Magenta GA-7 boxes (Magenta Corp., Chicago) that had been inoculated with *P. parasitica* 'Riverland'. In the preliminary study (Experiment 1), two 'Valencia' shoots were placed in inoculated medium and two were placed in uninoculated medium as controls. In Experiment 2, shoots of the cultivars 'Swingle' (2 shoots), Carrizo' (1 shoot), and 'Hamlin' (2 shoots) were excised and the bases of the stems were wounded by scalpel incisions before placement in the medium. Shoots were placed so that the wounded stem tissue remained in contact with the surface of the medium (where the *Phytophthora* grew most vigorously). The lengths of the resulting stem discoloration were recorded periodically during the following 12-24 days. Lesion growth rate was estimated by the slope of the linear regression of lesion length on time after placement for each cultivar.

For Experiment 3, six shoots from each of the cultivars 'Swingle', 'Carrizo', and 'Hamlin' were excised above the cotyledons 3-4 weeks after seed germination, and wounded at the base by making an elongated diagonal cut from one side of the stem to the other with a scalpel. The base of each shoot was then placed in solidified RM1 medium so that the wounded stem tissue remained in contact with the surface of the medium. The RM1 medium was inoculated with *P. parasitica* 'Riverland' a few days before stem placement. The lengths of the resulting stem discoloration were recorded 4, 6, 8, 10, and 12 days after stem placement.

Experiment 4. Outer seed coats were removed from seeds of 'Ridge Pineapple' and 'Valencia' sweet oranges (*C. sinensis*), 'Marsh' grapefruit (*C. ×paradisi*), Sour Orange selection Florida DPI-WH 13/12 (*C. aurantium* L.), 'Cleopatra' mandarin (*C. reticulata* Blanco), Volkamer lemon (*C. volkameriana* Ten. & Pasq., or *C. limon* [L.] Burm.f. 'Volkamer'), 'Carrizo' citrange, 'Swingle' citrumelo, *P. trifoliata* selection Florida DPI-WH 9/6, and *Severinia buxifolia* (Poir.) Tenore. Embryos were surface sterilized by a 30 second wash in 70% ethyl alcohol followed by immersion and agitation in 15% bleach (0.8% sodium hypochlorite plus 2 drops Tween per 100 ml) for 15 minutes. The seeds were then rinsed in sterile distilled water five times (five minutes each rinse), and individual seeds were placed on the surface of 10 ml GM1 medium solidified in the bottom of 25 × 150 mm glass culture tubes. The tubes were covered with translucent plastic Kap-uts and sealed with Nescofilm (Nippon Shoji Kaisha Ltd., Osaka, Japan). Seedling and dual cultures were maintained at 27°C in continuous darkness.

After shoots of each cultivar exceeded 70 mm in length (5-6 weeks from seed placement in tubes), they were excised above the cotyledons and 3-5 vertical slices made in the basal end of each shoot, leaving longitudinal flaps of stem tissue attached to the base of the stem. The basal end of each stem was then inserted several millimeters into solidified GM1 medium at the actively growing edge of a *P. parasitica* 'Hall' culture in a Magenta box. The medium in each Magenta box was inoculated with *P. parasitica* 'Hall' about five days prior to shoot placement in the medium. Shoots were arranged so that one shoot of each of the ten cultivars was in each of six Magenta boxes. The lengths of the resulting stem discoloration were recorded 2, 3, 4, 5, and 6 days after placement.

Results and Discussion

Phytophthora parasitica 'Hall' grew on corn meal agar as an expanding circle that spread out from the point of inoculation at a relatively uniform rate. Measurements of the distance (in mm) from point of inoculation to the margins of the *Phytophthora* growth were completed at 2, 3, 4, 5, and 6 days after inoculation on three Petri plates of corn meal agar. The rate of *Phytophthora* growth on this medium could then be estimated by linear regression of distance on time. The slope of the regression line indicated that the *P. parasitica* 'Hall' growth rate was about 3.8 $mm \cdot day^{-1}$ on BBL corn meal agar (at 27°C).

During Experiment 1, *P. parasitica* 'Riverland' was observed to invade excised green shoots of three-month-old 'Valencia' seedlings in dual cultures. Invasion of the stems by *Phytophthora* could be observed

as a distinct browning that moved from the agar surface (and shoot base) towards the shoot apex at a relatively constant rate. The mycelium of the fungus was rarely visible on the exterior of green shoots during the in vitro testing, and then only as small tufts at the nodes or the severed shoot apex. Growth of the mycelium from browned shoots on corn meal agar following the test indicated the presence of viable *Phytophthora* inoculum throughout the browned stems. Other green 'Valencia' shoots placed in uninoculated medium did not become discolored within 24 days. The rate of shoot browning or discoloration in response to *Phytophthora* infection was selected as one characteristic that may differ among genotypes and that may represent one aspect of relative cultivar susceptibility to the pathogen. This rate was estimated by measurement of the distance that the lesion (or discoloration) on a stem had progressed from the point of inoculation at several different periods of time after infection. Linear regression of these measurements produced a slope that should be a robust estimate of rate. This procedure also yielded a second characteristic for the regression line, the y-intercept, that may indicate other aspects of plant resistance to *Phytophthora* (such as the time after infection that the plant response was initiated). The y-intercept was not considered in the following discussions. Linear regression of lesion length (in mm) on days after placement of shoots on the inoculated medium produced estimates of 2.0 $mm \cdot day^{-1}$ for the slope of three-month-old, green 'Valencia' shoots. A later attempt to infect excised green 'Valencia' shoots was not successful and was interpreted as a failure of the *Phytophthora* to invade the stem without an exposed wound surface. This prompted the wounding of the stem tissue at the medium surface for the ensuing experiments (2-4).

During Experiment 2, *P. parasitica* 'Riverland' caused the progressive browning of wounded two-week-old green stems of the cultivars 'Hamlin' (two shoots), 'Carrizo' (one shoot), and 'Swingle' (two shoots). Estimates of slope were obtained by linear regression of lesion length (in mm) on time after placement for each cultivar. Mean slope values are shown in Table 6.1. If the rate of green stem browning in response to infection by *Phytophthora* (as measured by slope) is an indication of cultivar susceptibility, then the results of Experiment 2 indicated that 'Carrizo' was most susceptible, 'Hamlin' intermediate, and 'Swingle' the most resistant.

Table 6.1. Mean regression slopes for excised green shoot browning from *Phytophthora* and separation by Duncan's multiple range test ($\alpha = 0.05$).

Cultivar	Experiment 2 Regression slope	Experiment 3 Regression slope	Experiment 3 Duncan grouping
Swingle	1.5 mm·day^{-1}	1.5 mm·day^{-1}	a
Hamlin	2.7	2.6	ab
Carrizo	8.0	3.8	b

In Experiment 3, *P. parasitica* 'Riverland' caused the browning of wounded three- to four-week-old green stems of the cultivars 'Hamlin (six shoots), 'Carrizo' (six shoots), and 'Swingle' (six shoots). Slope for lesion growth rate was estimated by linear regression of lesion length (in mm) on time (4, 6, 8, 10, and 12 days) after placement in the medium for

each shoot. The individual estimates of slope for each of the six shoots per cultivar were used as replicates in an analysis of variance (ANOVA) procedure and Duncan's mean separation. The ANOVA procedure for slope indicated significant cultivar differences (the probability of a greater F was 0.015). Duncan's mean separations for calculated slope values are shown in Table 6.1. On the basis of rate of green stem tissue browning in response to *Phytophthora* infection, (i.e., slope) during Experiment 3, 'Carrizo' appeared to be the most susceptible, 'Hamlin' was intermediate, and 'Swingle' most resistant. This ranking of the response of green shoots to *Phytophthora* for the three cultivars was the same as in Experiment 2.

Browning response of etiolated seedlings following inoculation with *P. parasitica* 'Hall' varied considerably among the ten genotypes examined in Experiment 4. Regression of discoloration length on time indicated the response of most individual shoots was closely approximated by a straight line. The SAS PROC RSQUARE procedure was used to evaluate whether there was evidence that the cultivar responses had significant quadratic or cubic components. The six replicates for each cultivar were grouped together for this analysis. A linear model (independent variable = time in days; dependent variable = length in mm) produced the greatest or close to the greatest r^2 for each of the ten genotypes. Addition of quadratic ($time^2$) or cubic ($time^3$) components to the linear model produced insignificant increases in the r^2 value: The response was linear. The linear equations determined to best fit the response of each cultivar are given below, along with r^2 values.

Carrizo	$y = -17 + 10.4x$	$r^2 = 0.81$
Swingle	$y = -16 + 9.4x$	$r^2 = 0.81$
Valencia	$y = -6 + 7.7x$	$r^2 = 0.55$
Marsh	$y = -6 + 6.0x$	$r^2 = 0.50$
Sour	$y = -4 + 5.9x$	$r^2 = 0.27$
Cleopatra	$y = -5 + 5.8x$	$r^2 = 0.75$
Ridge	$y = -1 + 4.1x$	$r^2 = 0.34$
Volkamer	$y = 3.6x$	$r^2 = 0.48$
Poncirus	$y = 4 + 0.2x$	$r^2 = 0.04$
Severinia	$y = 3 + 0.1x$	$r^2 = 0.01$

Graphical representation of these equations is shown in Figure 6.1. A great difference between the response of the two *Citrus* relatives, *Poncirus* and *Severinia*, and the *Citrus* cultivars and hybrids was noted. ANOVA was performed, and LSD mean separations of cultivars were calculated, by using the slope of each of the six shoots per cultivar as replicates. The ANOVA procedure indicated highly significant cultivar differences for slope (the probability of a greater F was about 0.0001). Calculated values for slopes are shown along with LSD mean separations in Fig. 6.2.

At least three distinct types of etiolated shoot response to *Phytophthora* could be identified among the ten cultivars that were examined. The genotypes most resistant to *Phytophthora* were the *Citrus* relatives *Poncirus trifoliata* and *Severinia buxifolia*. These two selections exhibited very little discoloration of their stem tissue as indicated by the derived linear regression slope values of 0.2 $mm \cdot day^{-1}$ for *Poncirus* and 0.1 $mm \cdot day^{-1}$ for *Severinia*. This indication of very high

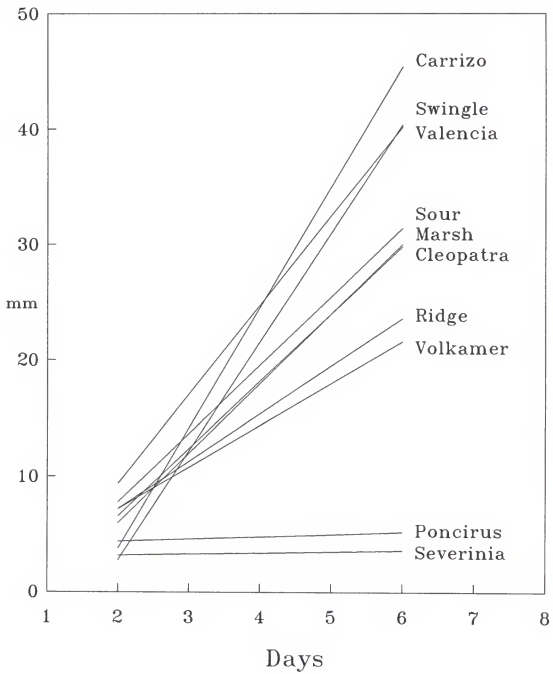

Figure 6.1. Estimated linear response, as discoloration length, of the ten cultivars to *Phytophthora* 2-6 days after inoculation.

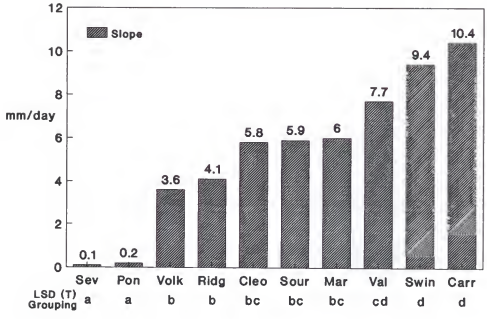

Figure 6.2. Mean regression slopes for excised etiolated shoot discoloration from *Phytophthora* and LSD comparisons (α = 0.05).

resistance or immunity to *P. parasitica* for *Severinia* was in agreement with field performance and the results of other tests (Carpenter and Furr, 1962; Grimm and Hutchison, 1977; Klotz and Calavan, 1978). *Poncirus trifoliata* is generally considered highly resistant or immune to *Phytophthora* as well (Timmer and Menge, 1988), although some variation in resistance has been reported among different *P. trifoliata* selections (Carpenter and Furr, 1962; Grimm and Hutchison, 1977; Univ. Calif., 1978). It appeared that the selection of *P. trifoliata* used in this test (Florida DPI-WH 9/6) was highly resistant to etiolated stem infection.

The *Citrus* cultivars Volkamer lemon, 'Ridge Pineapple', 'Cleopatra', sour orange, 'Marsh', and possibly 'Valencia' formed a group of selections

with intermediate response to *Phytophthora*. These cultivars produced a response with regression slopes in the range of 3.6 to 7.7 mm·day^{-1}. Although some statistically significant differences were detected within the group, retesting will be necessary to conclude that these differences were meaningful. Generally, Volkamer lemon, 'Cleopatra', and sour orange are considered to possess low to moderate levels of *Phytophthora* resistance, while sweet oranges ('Valencia' and 'Ridge Pineapple') and grapefruit ('Marsh') are considered highly susceptible (Castle, 1987; Timmer and Menge, 1988). However, considerable variability in *Phytophthora* susceptibility has been observed among different sour (Hutchison and Grimm, 1972) and sweet orange selections (Smith et al., 1987), so it is quite uncertain what would be the field performance of the clones used in the in vitro test. The failure of the in vitro test to discriminate clearly among these six intermediate cultivars may indicate that there was little difference among these cultivars in response to the specific test conditions. However, mechanisms of tolerance or resistance only activated in green seedling shoots or not measured by this test (such as feeder root regeneration and resistance to initial injury) may play important roles in relative resistance or tolerance of these cultivars to *Phytophthora* under field conditions.

The third type of response observed among the tested selections under these in vitro conditions was extreme susceptibility to *Phytophthora*. The response of 'Valencia' was overlapping with this group and the intermediate response described above. The rapid discoloration of etiolated stems of 'Carrizo' and 'Swingle' following inoculation with *Phytophthora* was indicated by regression slopes of 10.4 and 9.4 mm·day^{-1},

respectively. This result was unexpected because 'Swingle' has been considered highly tolerant or resistant to *Phytophthora* (Castle, 1987; Carpenter and Furr, 1962; Graham, in press; Timmer and Menge, 1988), while 'Carrizo' has been observed to have a lower, but still significant, degree of resistance or tolerance (Castle, 1987; Grimm and Hutchison, 1977; Timmer and Menge, 1988). The three in vitro tests involving these two cultivars (Experiments 2-4) did, however, agree with their relative field performance in that 'Swingle' was consistently more resistant than 'Carrizo' to discoloration from *Phytophthora*. The observed great susceptibility of 'Swingle' and 'Carrizo' during in vitro testing of etiolated shoots was especially surprising because these two cultivars are hybrids of *Poncirus trifoliata* (indicated to be highly resistant in this test) and were considered to have obtained their resistance to *Phytophthora* from this parent. The fact that both hybrids produce a similar response, however, reduces the probability that these unexpected results were due to experimental error. It is possible that this test of in vitro-grown etiolated shoots avoids the resistance mechanism(s) of 'Carrizo' and 'Swingle'. The resistance mechanisms in these selections appeared to be different from the predominant one(s) in *Poncirus* and may require light or greater seedling maturity for activation. The observation that non-etiolated 'Hamlin' sweet orange (considered highly susceptible to *Phytophthora*) was more resistant than non-etiolated 'Carrizo' (considered moderately resistant) in Experiments 2 and 3 supports the hypothesis that maturity may be an important factor controlling activation of resistance in 'Carrizo'. Graham (in press) reported a reduction in root rot among in vivo seedlings with increasing

age. However, a possible role for light in the activation process was suggested by the fact that green shoots of the cultivars 'Swingle' and 'Carrizo' both appeared considerably less susceptible to *Phytophthora* than when etiolated, as indicated by discoloration rate. It may be that both light and tissue maturity are involved in the activation of *Phytophthora* resistance in 'Carrizo' and 'Swingle'. Yet another explanation for the extreme sensitivity of 'Carrizo' and 'Swingle' to *P. parasitica* under these test conditions is that the resistance to *Phytophthora* observed in 'Carrizo' and 'Swingle' under field and other test conditions may be due to an ability to inhibit initial invasion of the plant tissues at wound sites. However, if the pathogen is able to penetrate this first line of defense under conditions especially favorable to *Phytophthora* (e.g. in vitro inoculation of severely wounded stems), and the host has limited physiological resources (e.g. no photosynthesis or root reserves), the remaining tissues will have little capacity to resist rampant *in planta* proliferation of *Phytophthora*.

Several aspects of these in vitro tests should be noted. First, two different isolates of *P. parasitica* were used because of contamination of all 'Riverland' cultures a few weeks before the initiation of Experiment 4. To avoid a long delay in reisolation of pure 'Riverland' cultures, a second isolate, 'Hall', was substituted in the experiment. Although different isolates of *P. parasitica* are not known to vary in virulence on *Citrus* (personal communication, J.H. Graham), it cannot be ruled out that isolate differences may have influenced cultivar responses. Second, the three cultivars used in the in vitro tests of green shoots (Experiments 2 & 3) proved to be the three most susceptible among those examined in the

etiolated test (assuming 'Hamlin' sweet orange = 'Valencia' sweet orange); Only preliminary conclusions can therefore be reached concerning the comparison of green to etiolated tissues. Third, growth of *Phytophthora* on excised shoots was usually restricted to the interior of the stem, and measurement of cultivar response was based entirely on stem discoloration (etiolated shoots) or browning (green shoots). Occasional small tufts of mycelium observed at nodes or the excised apex of infected discolored shoots, especially common in etiolated 'Carrizo' stems, suggested that discoloration or browning was providing a reasonable indication of *Phytophthora* movement through the tissues. In addition, short segments cut from aerial sections of browned shoots were reliable sources of *Phytophthora* inoculum. However, plant response (as discoloration or browning) was the measured parameter, and was not necessarily the same as the degree of *Phytophthora* colonization of the stem tissue.

This work demonstrated that two isolates of *Phytophthora parasitica* can successfully invade wounded excised shoots of *Citrus* seedlings in dual cultures. Rates of shoot discoloration were determined, in vitro, for etiolated seedling shoots of ten *Citrus* cultivars and relatives, and for green seedling shoots of three cultivars. For many cultivars, the observed relative responses to *Phytophthora* were in agreement with accepted field responses and other testing methods (e.g. *Poncirus*, *Severinia*, Volkamer lemon, 'Cleopatra', sour orange, 'Marsh', and 'Valencia'). In other cases, the observed response to *Phytophthora* was nearly opposite to known field resistance (e.g. 'Swingle'). The failure of the method to produce results entirely in agreement with field performance, as well as the unexpectedly large amount of work it requires,

makes it unlikely to be useful as a screening procedure in breeding efforts. However, other types of tissue may yield results that correlate better with field response. In addition, the method may provide a precise way of measuring some plant-pathogen interactions (e.g. hypersensitivity) under carefully controlled conditions for investigations of resistance mechanisms.

CHAPTER 7
SUMMARY AND CONCLUSIONS

The introductory chapter pointed out the achievements and potential of several approaches to *Citrus* genetic improvement. Regardless of the ultimate value of these methods, it seems certain that they will not solve all the problems facing contemporary citriculture. In addition, most of the popular approaches to cultivar development focus on methods of generating genetic changes and devote relatively few resources to improving techniques of germplasm evaluation. Considerable benefit could be derived from the development of other, as yet unexploited, sources of genetic variation and methods of evaluation. The research described in this dissertation has completed one stage of investigations on five topics in this category: a diverse semi-wild population with zygotic embryony as a source of potential breeding parents, the morphologic and potential genetic variation in plants regenerated from tissue culture, the response of tissue cultures to gamma irradiation as a source of mutations, fruit sector chimeras and recovery of mutant plants, and a method of testing in vitro response of *Citrus* selections to *P. parasitica*. The results from each of these projects were discussed in the respective chapters.

Programs in genetic improvement must consider many factors, including ease of propagation, horticultural performance, yield, and consumer acceptance. The breeder's job is complicated further by strict

limits of time and financial resources, making it impossible for one program to pursue more than a few potential avenues of genetic improvement during the career of a breeder. These limitations are more severe with crops having long juvenile periods, like *Citrus*. Consequently, difficult choices must be made about program direction(s) early in a breeder's career with only scant information and limited experience. Ideally, these decisions will be based on the costs of and potential returns from all the available options. Initial investigations, such as those described in this dissertation, may provide valuable information on which to base decisions about the relative expenses (time and money) and potential profit (in genetic advancement) of various approaches to genetic improvement and cultivar development. It may be prudent to devote a few years to evaluation of an approach under carefully controlled conditions (or with model genotypes) before proceeding with long term investments of time and money. With this perspective, a general assessment will be presented of the relative value of the several avenues for *Citrus* "breeding" that were described in this dissertation.

The connection between the Caribbean forbidden fruit and the grapefruit has been the source of considerable controversy over nearly two centuries. Although the rediscovery of the forbidden fruit in Saint Lucia sheds considerable insight into the sources of the confusion, it will probably not put the controversy to rest. The isozyme and morphological data indicated that forbidden fruit and grapefruit are very closely related, although the former is considerably more diverse in characteristics and has a much higher degree of zygotic embryony. These same two characteristics (along with a possible greater degree of pollen

fertility) make the forbidden fruit a promising parent for use in sexual hybridization, regardless of its actual taxonomic standing. In this respect, the forbidden fruit is not a new breeding direction, but a small (although potentially valuable) addition to the available *Citrus* breeding germplasm. A second aspect of the forbidden fruit potential that deserves mention is the possibility for the development of a new *Citrus* fruit type for fresh marketing. Most Saint Lucian forbidden fruit selections have good flavor characteristics that resemble grapefruit but are distinct in some respects as well. Many of the forbidden fruit selections appear somewhat intermediate between grapefruit and pummelo, and may present a pummelo-like form that could win acceptance in the American marketplace. The same attraction to the "forbidden" that resulted in biblical mankind's expulsion from the Garden of Eden may, with proper promotional titillation, provide an urge to consume the contemporary forbidden fruit that would reserve for it a place on the specialty produce counter.

Generation of variation by tissue culture (somaclonal variation) has been the subject of research reports totaling in the hundreds over the past decade. Many of these reports have expounded a tremendous potential for the technique in pursuit of genetic improvement. Without question the phenomenon does occur in many plant species and in *Citrus*. However, the morphological evaluation of 'Carrizo' and 'Hamlin' somaclones regenerated by organogenesis indicated that stable aberrant plants are not frequently produced. Considering the relatively great investment of time required to produce somaclones by this method, and the expectation that most mutations will be deleterious, this probably will not be a cost effective method of obtaining useful genetic variation. For this reason,

preliminary information was gathered on gamma irradiation of tissue cultures as a supplemental source of mutagenesis. A much higher frequency of genetic variants can be expected among regenerates from gamma irradiated tissue cultures. Irradiation or other mutagenic treatments are, therefore, advisable for use with tissue cultures as a source of *Citrus* genetic mutants.

Sectored chimeric citrus fruit were easily recovered from packinghouses. Observations of potential genetic improvements in some sector mutants and successful recovery of mutations in nucellar seedlings from sectors suggest that sectored fruit may be a valuable source of genetic mutants. This resource is particularly attractive because of the ability to select desirable mutants at the start of the research involvement (the packinghouse). Preliminary contacts with packinghouse management indicated that it would be possible to have the packinghouse graders preselect any desired type of sectored fruit. The breeder could then select from these elite fruit the more promising candidates for propagation and field trials. The types of mutations would, of course, generally be restricted to those that could be observed in the fruit rind. However, these are the kinds of genetic changes that are often of most economic significance and impossible to select for at the seedling stage. Preliminary indications are that fruit sector chimeras are a very inexpensive (in both time and money) source of "selected mutations" that have considerable promise for genetic improvement of existing cultivars. One class of sector chimeras has already provided tetraploid clones that may be used in breeding.

The response of etiolated seedling shoots to *P. parasitica* in vitro appeared to be a poor method of selecting genotypes resistant to foot rot or root rot in a breeding program, at least among trifoliate hybrids. The observation that the field-resistant cultivar, 'Swingle', exhibited a susceptible response under the test conditions indicated that the test results did not correlate well with field response. Preliminary evidence suggested that green seedling shoots may give a more useful indication of resistance, but the results were inconclusive. At any rate, this method appeared to be too expensive (in labor) to use with large numbers of genotypes. However, the technique may hold promise for controlled studies of resistance mechanisms.

Success in breeding/genetic improvement, in contrast to "pure" science, must be measured in practical terms. Techniques producing spectacular results or novel genotypes do not equate with breeding progress. Similarly, mundane procedures or an accident of nature may result in a genetic combination that will dominate an industry for decades or centuries. The 'Washington' navel and 'Valencia' sweet oranges are examples of cultivars with tremendous commercial value that arose by chance (Hodgson, 1967). A program for genetic improvement of *Citrus* should employ the most efficient and promising techniques to achieve the desired goals. However, a *Citrus* breeder should recognize that favorable genetic combinations will be determined primarily by chance even in the most carefully designed and executed programs for genetic improvement. The ability to recognize useful genetic combinations wherever they occur and accurately select the best from the millions will probably remain the ultimate determinant of the pace of genetic advancement.

APPENDIX

Table A.1. Adaxial leaf color of selected control genotypes with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Adaxial leaf color		
		A	B	L
HAMLIN MERICLONE	62	-13.58	17.12	37.00
Valencia	58	-14.81*	19.67*	39.94
Duncan/Z	5	-12.90	16.08	35.42
SF23-1	5	-10.24**	10.77**	29.67**
SF24-1	16	-12.23*	13.92*	33.35*
Marsh	5	-15.10**	20.69**	42.50**
Meiwa	0	nd	nd	nd

* = mean significantly different from HAMLIN MERICLONE with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$;
N = number of observations; nd = no data.

Table A.2. Abaxial leaf color of selected control genotypes with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Abaxial leaf color A	B	L
HAMLIN MERICLONE	62	-14.25	21.34	49.91
Valencia	57	-14.23	22.07	52.27*
Duncan/Z	5	-14.27	22.39*	50.52
SF23-1	5	-14.22	21.36	48.24
SF24-1	16	-14.36	22.49**	51.05
Marsh	5	-14.62	23.84**	55.21**
Meiwa	0	nd	nd	nd

* = mean significantly different from HAMLIN MERICLONE with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$;
N = number of observations; nd = no data.

Table A.3. Leaf color ratios of selected control genotypes with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Leaf color as A/B	
		Adaxial	Abaxial
HAMLIN MERICLONE	62	-0.8016	-0.6684
Valencia	57	-0.7604	-0.6455*
Duncan/Z	5	-0.8056	-0.6375**
SF23-1	5	-0.9522**	-0.6657
SF24-1	16	-0.8810**	-0.6387**
Marsh	5	-0.7328*	-0.6135**
Meiwa	0	nd	nd

* = mean significantly different from HAMLIN MERICLONE with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$;
N = number of observations; nd = no data.

Table A.4. Spine size and shape of selected control genotypes with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Spine length	Spine dia.	Spine L/D
HAMLIN MERICLONE	67	26 mm	1.2 mm	23
Valencia	60	30	1.2	25
Duncan/Z	6	18**	1.4**	13**
SF23-1	8	15**	1.1	14**
SF24-1	20	14**	1.1	12**
Marsh	7	13**	1.0	11**
Meiwa	3	14**	1.2	11**

* = mean significantly different from HAMLIN MERICLONE with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$;
N = number of observations; nd = no data.

Table A.5. Leaf size and shape of selected control genotypes with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Leaf length	Leaf width	Leaf L/W
HAMLIN MERICLONE	67	43 mm	27 mm	1.6
Valencia	60	46	27	1.7
Duncan/Z	5	50	34**	1.5
SF23-1	5	35*	21*	1.7
SF24-1	18	37	23	1.6
Marsh	5	44	26	1.7
Meiwa	3	46	18**	2.5**

* = mean significantly different from HAMLIN MERICLONE with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$;
N = number of observations; nd = no data.

Table A.6. Leaf oil gland density and internode length of selected control genotypes with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Oil glands per 20 mm^2	Internode length
HAMLIN MERICLONE	65	29	17 mm
Valencia	60	32	18
Duncan/Z	5	10**	13
SF23-1	8	10**	8**
SF24-1	20	16*	9**
Marsh	5	21	10*
Meiwa	3	131**	8*

* = mean significantly different from HAMLIN MERICLONE with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$;
N = number of observations; nd = no data.

Table A.7. Adaxial leaf color of somaclones with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Adaxial leaf color A	B	L
HAMLIN MERICLONE	62	-13.58	17.12	37.00
HA142E121	2	-9.73**	10.95**	29.83**
HA142E122	5	S	S	S
HA142E124	5	S	S	S
HA142E211	4	S	S	*
HA142E215	5	S	S	S
HA142E221	1	S	S	S
HA142E222	1	*	S	S
HA142E251	3	S	S	S
HA142E252	3	S	S	S
HA151E121	3	S	S	S
HA151E122	5	S	S	S
HA4E121	4	S	S	*
HA4I221	4	S	S	S
HA5E121	1	S	*	*
HA5I122	3	S	S	S
HA5I123	5	S	S	S
HA5I124	3	*	*	S
HA6E112/Z	5	S	S	S
HA6E114/Z	3	*	22.86**	46.26**
HA6E116/Z	5	S	*	*
HA7E11/Z	5	S	S	S
HA7E12/Z	5	S	S	S

Table A.7. (continued)

Clone	N	Adaxial leaf color A	B	L
HA9E13/Z	3	S	*	*
HX1X11	3	S	S	S
HX1X12	3	S	*	29.83**
HX101E111	3	S	S	S
HX111E11	5	S	S	S
HX115E12/Z	3	S	S	S
HX115E14/Z	5	S	*	*
HX14E12	4	S	S	S
HX15E11	5	S	S	S
HX20E11	5	-16.33**	23.15**	46.26**
HX21E11	5	S	S	S
HX22I11	4	S	S	S
HX50E13	5	S	S	S
HX60E11	5	*	*	*
HX61E11	3	S	S	S
VA13E11	1	S	S	S
VA30E11	4	S	S	S
VA33E11	5	S	*	S
VX91E11/Z	5	*	22.50**	44.33**
VX91E12/Z	5	S	S	S
ANOVA GLM PR>F (df=42)		0.0001	0.0001	0.0001

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.8. Abaxial leaf color of somaclones with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Abaxial leaf color A	B	L
HAMLIN MERICLONE	62	-14.25	21.34	49.91
HA142E121	2	-13.16**	19.49**	45.39**
HA142E122	5	S	S	S
HA142E124	5	S	S	S
HA142E211	4	S	S	S
HA142E215	5	S	S	S
HA142E221	1	S	S	S
HA142E222	0	nd	nd	nd
HA142E251	3	S	S	S
HA142E252	3	S	S	S
HA151E121	3	S	S	S
HA151E122	5	S	S	S
HA4E121	4	S	S	S
HA4I221	4	*	S	S
HA5E121	1	S	S	S
HA5I122	3	S	S	S
HA5I123	5	S	S	S
HA5I124	3	S	S	S
HA6E112/Z	5	S	S	S
HA6E114/Z	3	S	*	54.03**
HA6E116/Z	5	S	S	*
HA7E11/Z	5	S	S	S
HA7E12/Z	5	S	*	*

Table A.8. (continued)

Clone	N	Abaxial leaf color A	B	L
HA9E13/Z	3	S	*	54.46**
HX1X11	3	S	S	S
HX1X12	3	S	19.39**	45.23**
HX101E111	3	S	S	S
HX111E11	5	S	*	S
HX115E12/Z	3	S	S	S
HX115E14/Z	5	S	*	54.22**
HX14E12	4	S	S	S
HX15E11	5	S	S	S
HX20E11	5	*	23.14**	55.29**
HX21E11	5	S	S	S
HX22I11	4	S	S	S
HX50E13	5	S	S	S
HX60E11	5	S	S	*
HX61E11	3	S	S	S
VA13E11	1	*	S	S
VA30E11	4	S	S	S
VA33E11	5	S	*	*
VX91E11/Z	5	S	*	54.93**
VX91E12/Z	5	S	*	*
ANOVA GLM PR>F (df=41)		0.0001	0.0001	0.0001

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.9. Leaf color ratios of somaclones with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Leaf color as A/B	
		Adaxial	Abaxial
HAMLIN MERICLONE	62	-0.8016	-0.6684
HA142E121	2	S	S
HA142E122	5	S	S
HA142E124	5	S	S
HA142E211	4	S	S
HA142E215	5	S	S
HA142E221	1	S	*
HA142E222	1	S	nd
HA142E251	3	S	S
HA142E252	3	S	S
HA151E121	3	*	*
HA151E122	5	S	S
HA4E121	4	S	S
HA4I221	4	S	S
HA5E121	1	-0.9929**	S
HA5I122	3	S	S
HA5I123	5	S	S
HA5I124	3	S	S
HA6E112/Z	5	S	S
HA6E114/Z	3	*	S
HA6E116/Z	5	*	S
HA7E11/Z	5	S	S
HA7E12/Z	5	S	-0.6166**

Table A.9. (continued)

Clone	N	Leaf color as A/B Adaxial	Abaxial
HA9E13/Z	3	*	-0.6127**
HX1X11	3	S	S
HX1X12	3	-0.9290**	-0.7190**
HX101E111	3	S	S
HX111E11	5	S	*
HX115E12/Z	3	S	S
HX115E14/Z	5	*	-0.6241**
HX14E12	4	S	S
HX15E11	5	S	S
HX20E11	5	*	S
HX21E11	5	S	S
HX22I11	4	S	S
HX50E13	5	S	S
HX60E11	5	S	S
HX61E11	3	S	S
VA13E11	1	S	S
VA30E11	4	S	*
VA33E11	5	S	-0.6280**
VX91E11/Z	5	*	*
VX91E12/Z	5	S	*
ANOVA GLM PR>F (df=42)		0.0001	0.0001

S = mean not different from HAMLIN MERICLONE with α = 0.05;
* = mean significantly different with α = 0.05;
** = mean significantly different with α = 0.01; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.10. Spine size and shape of somaclones with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Spine length	Spine dia.	Spine L/D
HAMLIN MERICLONE	67	26 mm	1.2 mm	23
HA142E121	4	S	S	*
HA142E122	6	S	S	S
HA142E124	5	S	S	S
HA142E211	5	S	S	S
HA142E215	5	S	S	15**
HA142E221	2	S	S	S
HA142E222	2	S	S	*
HA142E251	5	S	S	S
HA142E252	5	S	S	S
HA151E121	5	S	S	S
HA151E122	5	S	S	S
HA4E121	5	S	S	S
HA4I221	5	S	0.9**	S
HA5E121	3	*	0.9**	15**
HA5I122	5	10**	0.8**	16**
HA5I123	2	S	S	S
HA5I124	4	S	S	S
HA6E112/Z	5	S	S	S
HA6E114/Z	5	S	S	S
HA6E116/Z	5	S	S	S
HA7E11/Z	5	S	S	S
HA7E12/Z	5	S	S	S

Table A.10. (continued)

Clone	N	Spine length	Spine dia.	Spine L/D
HA9E13/Z	5	S	S	S
HX1X11	4	*	0.9**	*
HX1X12	3	9**	0.8**	16**
HX101E111	5	S	S	S
HX111E11	5	S	S	S
HX115E12/Z	5	S	S	S
HX115E14/Z	5	S	S	S
HX14E12	5	S	S	S
HX15E11	5	S	S	S
HX20E11	5	S	S	S
HX21E11	5	S	S	S
HX22I11	5	S	S	S
HX50E13	5	S	S	S
HX60E11	5	S	S	S
HX61E11	5	S	S	S
VA13E11	3	S	S	S
VA30E11	5	*	S	S
VA33E11	5	S	S	S
VX91E11/Z	5	*	*	*
VX91E12/Z	5	S	S	S
ANOVA GLM PR>F (df=42)		0.0001	0.0001	0.0001

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.11. Leaf size and shape of somaclones with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Leaf length	Leaf width	Leaf L/W
HAMLIN MERICLONE	67	43 mm	27 mm	1.6
HA142E121	4	S	*	S
HA142E122	5	S	S	S
HA142E124	5	S	S	S
HA142E211	5	S	S	S
HA142E215	5	S	S	S
HA142E221	2	S	*	*
HA142E222	2	S	19**	S
HA142E251	5	S	S	S
HA142E252	5	S	S	S
HA151E121	5	S	S	S
HA151E122	5	S	S	S
HA4E121	5	S	S	S
HA4I221	5	S	18**	S
HA5E121	3	S	*	S
HA5I122	5	S	S	1.9**
HA5I123	5	S	S	S
HA5I124	3	S	S	S
HA6E112/Z	5	S	S	S
HA6E114/Z	5	S	S	S
HA6E116/Z	5	S	S	S
HA7E11/Z	5	S	S	S
HA7E12/Z	5	S	S	S

Table A.11. (continued)

Clone	N	Leaf length	Leaf width	Leaf L/W
HA9E13/Z	5	S	S	S
HX1X11	3	S	S	S
HX1X12	3	S	S	S
HX101E111	5	*	19**	S
HX111E11	5	S	S	S
HX115E12/Z	5	S	S	S
HX115E14/Z	5	S	S	S
HX14E12	5	S	S	S
HX15E11	5	S	S	S
HX20E11	5	S	S	S
HX21E11	5	S	S	S
HX22I11	5	S	S	S
HX50E13	5	S	S	S
HX60E11	5	S	S	S
HX61E11	5	S	*	S
VA13E11	3	S	*	S
VA30E11	5	S	S	1.9**
VA33E11	5	S	S	1.9**
VX91E11/Z	5	S	S	S
VX91E12/Z	5	S	S	S
ANOVA GLM PR>F (df=42)		0.0001	0.0001	0.0001

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.12. Leaf oil gland density and internode length of somaclones with mean comparison to HAMLIN MERICLONE by Duncan's multiple range test.

Clone	N	Oil glands per 20 mm^2	Internode length
HAMLIN MERICLONE	65	29	17 mm
HA142E121	3	S	S
HA142E122	5	S	S
HA142E124	4	S	S
HA142E211	5	S	S
HA142E215	5	S	S
HA142E221	1	S	S
HA142E222	1	S	S
HA142E251	5	S	S
HA142E252	5	S	S
HA151E121	4	S	S
HA151E122	4	S	S
HA4E121	5	S	S
HA4I221	3	S	S
HA5E121	2	S	S
HA5I122	5	S	S
HA5I123	1	S	S
HA5I124	4	S	S
HA6E112/Z	5	S	S
HA6E114/Z	5	S	S
HA6E116/Z	5	S	S
HA7E11/Z	4	S	S
HA7E12/Z	5	S	S

Table A.12. (continued)

Clone	N	Oil glands per 20 mm^2	Internode length
HA9E13/Z	5	S	S
HX1X11	3	S	S
HX1X12	2	*	S
HX101E111	5	S	S
HX111E11	5	S	S
HX115E12/Z	5	S	S
HX115E14/Z	5	S	S
HX14E12	5	S	S
HX15E11	5	S	S
HX20E11	5	S	S
HX21E11	5	S	S
HX22I11	5	S	S
HX50E13	5	S	S
HX60E11	5	S	S
HX61E11	5	S	S
VA13E11	3	S	S
VA30E11	5	S	S
VA33E11	4	S	S
VX91E11/Z	5	S	S
VX91E12/Z	5	S	S
ANOVA GLM PR>F (df=42)		0.0002	0.0030

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.13. Adaxial leaf color of selected somaclones during second test with mean comparison to MERICLONE by Duncan's multiple range test.

Clone	N	Adaxial leaf color		
		A	B	L
MERICLONE	57	-10.36	11.61	30.69
HA142E215	5	S	S	S
HA4E121	5	S	S	S
HA7E11/Z	5	S	S	S
HA7E12/Z	5	-14.08**	18.90**	39.36**
HA9E13/Z	5	S	S	S
HX1X11	5	S	S	S
HX1X12	3	S	S	S
HX101E111	5	S	S	S
HX111E11	5	S	S	S
HX115E12/Z	5	-13.42**	17.93**	38.18**
HX115E14/Z	5	S	S	S
HX15E11	3	-6.72**	*	*
HX20E11	5	S	S	S
HX22I11	5	S	S	S
HX61E11	5	S	S	*
ANOVA GLM PR>F (df=15)		0.0001	0.0001	0.0001
Number of 15 somaclones				
Differing at .05		3	3	4
Differing at .01		3	2	2

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.14. Abaxial leaf color of selected somaclones during second test with mean comparison to MERICLONE by Duncan's multiple range test.

Clone	N	Abaxial leaf color A	B	L
MERICLONE	57	-12.89	19.64	47.54
HA142E215	5	S	S	S
HA4E121	5	S	S	S
HA7E11/Z	5	*	S	S
HA7E12/Z	5	S	22.14**	53.90**
HA9E13/Z	5	S	S	S
HX1X11	5	S	S	S
HX1X12	3	S	S	S
HX101E111	5	S	S	S
HX111E11	5	S	S	S
HX115E12/Z	5	S	22.33**	54.36**
HX115E14/Z	5	S	S	S
HX15E11	3	S	S	S
HX20E11	5	S	S	S
HX22I11	5	S	S	S
HX61E11	5	S	*	*
ANOVA GLM PR>F (df=15)		0.0258	0.0001	0.0001
Number of 15 somaclones Differing at .05		1	3	3
Differing at .01		0	2	2

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.15. Leaf color ratios of HAMLIN MERICLONE and selected somaclones during second test with mean comparison to MERICLONE by Duncan's multiple range test.

Clone	N	Leaf color as A/B ratio Adaxial	Abaxial
MERICLONE	57	-0.9099	-0.6577
HA142E215	5	S	S
HA4E121	5	S	S
HA7E11/Z	5	S	S
HA7E12/Z	5	-0.7464**	-0.5821**
HA9E13/Z	5	S	S
HX1X11	5	S	S
HX1X12	3	S	S
HX101E111	5	S	S
HX111E11	5	S	S
HX115E12/Z	5	-0.7500**	-0.5792**
HX115E14/Z	5	S	S
HX15E11	3	S	S
HX20E11	5	S	S
HX22I11	5	S	S
HX61E11	5	S	S
ANOVA GLM PR>F (df=15)		0.0001	0.0001
Number of 15 somaclones Differing at .05		2	2
Differing at .01		2	2

S = mean not different from HAMLIN MERICLONE with α = 0.05;
* = mean significantly different with α = 0.05;
** = mean significantly different with α = 0.01; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.16. Spine size and shape of selected somaclones during second test with mean comparison to MERICLONE by Duncan's multiple range test.

Clone	N	Spine size and shape		
		Length	Diameter	L/D ratio
MERICLONE	56	29 mm	1.1 mm	26
HA142E215	5	15**	0.8**	19**
HA4E121	5	S	*	S
HA7E11/Z	5	*	*	S
HA7E12/Z	5	*	S	*
HA9E13/Z	5	S	S	S
HX1X11	4	S	S	S
HX1X12	3	*	S	18**
HX101E111	5	S	1.4**	S
HX111E11	5	S	S	S
HX115E12/Z	5	S	S	S
HX115E14/Z	5	S	*	S
HX15E11	3	*	*	*
HX20E11	5	S	S	S
HX22I11	5	S	S	S
HX61E11	5	S	S	S
ANOVA GLM PR>F (df=15)		0.0001	0.0001	0.0001
Number of 15 somaclones				
Differing at .05		5	6	4
Differing at .01		1	2	2

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.17. Leaf size and shape of selected somaclones during second test with mean comparison to MERICLONE by Duncan's multiple range test.

Clone	N	Leaf size and shape		
		Length	Width	L/W ratio
MERICLONE	58	63 mm	39 mm	1.6
HA142E215	5	S	*	*
HA4E121	5	S	S	S
HA7E11/Z	5	S	S	S
HA7E12/Z	5	S	S	S
HA9E13/Z	5	S	S	S
HX1X11	5	S	S	S
HX1X12	3	S	S	S
HX101E111	5	S	S	S
HX111E11	5	S	S	S
HX115E12/Z	5	S	S	S
HX115E14/Z	5	S	S	S
HX15E11	3	S	S	S
HX20E11	5	S	S	S
HX22I11	5	S	S	S
HX61E11	5	S	S	S
ANOVA GLM PR>F (df=15)		0.2578	0.0987	0.0081
Number of 15 somaclones				
Differing at .05		0	1	1
Differing at .01		0	0	0

S = mean not different from HAMLIN MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.18. Leaflet lengths of Carrizo mericlones, adventitious seedlings, and somaclones with mean comparison to Z2 MERICLONE by Duncan's multiple range test.

Clone	N	Leaflet length		
		Left	Main	Right
Z2 MERICLONE	12	22 mm	36 mm	22 mm
Z1 mericlone	6	S	S	S
Z3 mericlone	6	28**	S	28**
Z11 seedling	6	S	S	S
Z12 seedling	6	S	S	S
Z13 seedling	6	S	S	S
Z14 seedling	6	S	S	S
Z15 seedling	6	S	S	S
Z17 seedling	6	S	S	S
Z19 seedling	6	S	S	S
Z2E11	6	S	S	S
Z2E1CB111	6	S	S	S
Z2E1CB112	6	S	S	S
Z2E1CB113	6	S	S	S
Z2E1CB114	6	S	S	S
Z2E1CB231	6	S	S	S
Z2E1CB232	6	S	S	S
Z2E1CB315	6	S	S	S
Z2E1CB316	6	S	S	S
Z2E1CB317	6	16**	*	16**
Z2E21	6	S	S	S
Z2E22	6	S	S	S
Z2E211	6	S	S	S

Table A.18. (continued)

Clone	N	Leaflet length		
		Left	Main	Right
Z2E212	6	S	S	S
Z2E233	6	S	S	S
Z2E234	6	S	S	S
Z2E235	6	S	S	S
Z2E236	6	S	S	S
Z2E237	6	*	S	S
Z2E238	6	S	S	S
Z2E239	6	S	S	S
Z2E240	6	S	S	S
Z2E241	6	S	S	S
Z2E242	6	S	S	S
Z2E243	6	S	S	S
Z2E244	6	S	S	S
Z2I11	6	S	S	S
Z2I152	6	S	S	S
Z2I153	6	S	S	S
Z2I154	6	S	S	S
Z2I155	6	S	S	S
Z2I156	6	S	S	S
Z2I157	6	S	S	S
Z2I158	6	*	S	S
Z2I160	6	S	S	S
Z2I161	6	S	S	*

Table A.18. (continued)

Clone	N	Leaflet length		
		Left	Main	Right
Z2I162	6	S	S	S
Z2I163	6	S	S	S
Z2I164	6	S	S	S
Z2I165	6	S	S	S
Z2I166	6	S	S	S
Z2I167	6	S	S	S
Z2I168	6	S	S	S
Z2I21	6	S	S	S
Z2I269	6	S	S	S
Z2I270	6	S	S	S
Z2I271	6	S	S	S
ANOVA GLM PR>F (df=56)		0.0001	0.0001	0.0001
Number of somaclones				
Differing at .05		3	1	2
Differing at .01		1	0	1

S = mean not different from Z2 MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.19. Spine size and shape of 'Carrizo' mericlones, adventitious seedlings, and somaclones with mean comparison to Z2 MERICLONE by Duncan's multiple range test.

Clone	N	Spine length	Spine diameter	Spine L/D
Z2 MERICLONE	6	22 mm	1.2 mm	18
Z1 mericlone	6	S	*	S
Z3 mericlone	6	S	1.5**	S
Z14 seedling	6	S	1.4**	S
Z15 seedling	6	S	S	S
Z17 seedling	6	S	S	S
Z19 seedling	6	S	S	S
Z2E11	6	S	*	S
Z2E1CB111	6	S	S	S
Z2E1CB112	6	S	*	S
Z2E1CB113	6	S	*	S
Z2E1CB114	6	S	S	S
Z2E1CB231	6	S	*	S
Z2E1CB232	6	S	S	S
Z2E1CB315	6	S	S	S
Z2E1CB316	6	S	S	S
Z2E21	6	S	S	S
Z2E22	6	S	S	S
Z2E211	6	S	S	S
Z2E212	6	S	*	S

Table A.19. (continued)

Clone	N	Spine length	Spine diameter	Spine L/D
Z2E233	6	S	S	S
Z2E234	6	S	S	S
Z2E235	6	S	1.4**	S
Z2E236	6	S	1.4**	S
Z2E237	6	S	*	S
Z2E238	6	S	S	S
Z2E239	6	11**	1.0**	11**
Z2E240	6	S	1.4**	S
Z2E241	6	S	S	S
Z2E242	6	S	S	S
Z2E243	6	S	*	S
Z2I152	6	S	S	S
Z2I153	4	S	1.4**	S
Z2I154	6	S	S	S
Z2I155	6	S	S	S
Z2I157	6	*	1.0**	*
Z2I158	6	S	S	S
Z2I160	6	S	S	S
Z2I161	6	S	S	S
Z2I162	6	S	S	S

Table A.19. (continued)

Clone	N	Spine length	Spine diameter	Spine L/D
Z2I163	6	S	S	S
Z2I164	6	S	S	S
Z2I165	6	S	S	S
Z2I166	6	S	1.3**	S
Z2I167	1	10**	0.8**	12**
Z2I168	6	S	S	S
Z2I21	6	S	S	S
Z2I271	6	S	*	S
ANOVA GLM PR>F (df=47)		0.0001	0.0001	0.0012
Number of somaclones				
Differing at .05		3	19	3
Differing at .01		2	8	2

S = mean not different from Z2 MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

Table A.20. Leaflet ratio and chirality reversals of 'Carrizo' mericlones, adventitious seedlings, and somaclones with mean comparison to Z2 MERICLONE by Duncan's multiple range test.

Clone	N	Leaflet ratio	Chirality reversals
Z2 MERICLONE	12	1.2	0
Z1 mericlone	6	S	0
Z3 mericlone	6	S	0
Z11 seedling	6	S	3
Z12 seedling	6	S	0
Z13 seedling	6	S	1
Z14 seedling	6	S	2
Z15 seedling	6	S	0
Z17 seedling	6	S	0
Z19 seedling	6	S	2
Z2E11	6	S	1
Z2E1CB111	6	S	0
Z2E1CB112	6	S	3
Z2E1CB113	6	S	3
Z2E1CB114	6	S	0
Z2E1CB231	6	S	4*
Z2E1CB232	6	S	2
Z2E1CB315	6	S	1
Z2E1CB316	6	S	3
Z2E1CB317	6	*	0
Z2E21	6	S	0

Table A.20. (continued)

Clone	N	Leaflet ratio	Chirality reversals
Z2E22	6	S	0
Z2E211	6	S	2
Z2E212	6	S	0
Z2E233	6	S	0
Z2E234	6	S	0
Z2E235	6	S	2
Z2E236	6	S	1
Z2E237	6	S	0
Z2E238	6	S	0
Z2E239	6	*	0
Z2E240	6	S	4*
Z2E241	6	S	0
Z2E242	6	S	0
Z2E243	6	S	3
Z2E244	6	S	0
Z2I11	6	S	0
Z2I152	6	S	0
Z2I153	6	S	0
Z2I154	6	S	0
Z2I155	6	S	0

Table A.20. (continued)

Clone	N	Leaflet ratio	Chirality reversals
Z2I156	6	S	0
Z2I157	6	S	0
Z2I158	6	S	1
Z2I160	6	S	0
Z2I161	6	S	2
Z2I162	6	S	0
Z2I163	6	S	1
Z2I164	6	S	1
Z2I165	6	S	0
Z2I166	6	S	0
Z2I167	6	S	0
Z2I168	6	S	0
Z2I21	6	S	0
Z2I269	6	S	1
Z2I270	6	S	0
Z2I271	5	S	2
ANOVA GLM PR>F (df=56)		0.0006	-
Number of somaclones			
Differing at .05		2	2
Differing at .01		0	0

S = mean not different from Z2 MERICLONE with $\alpha = 0.05$;
* = mean significantly different with $\alpha = 0.05$;
** = mean significantly different with $\alpha = 0.01$; nd = no data;
N = number of observations; df = degrees of freedom.

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BIOGRAPHICAL SKETCH

Kim Dean Bowman was born May 2nd, 1957, in Dayton, Ohio, and completed his primary and secondary education in the nearby Brookville school system. He graduated from Brookville High School in 1975 as salutatorian and a member of the National Honor Society. After two years' employment in landscaping and factories, he enrolled in Miami University, Oxford, Ohio. He received the Bachelor of Science degree from Miami University in 1981 summa cum laude with honors in botany.

Kim Dean Bowman was awarded a Liberty Hyde Bailey Fellowship from Cornell University in 1981, where he began his graduate studies in the Department of Pomology. During his studies at Cornell University, he undertook research on breeding for resistance to the woolly apple aphid, completed the 1982 Athens Marathon in 2:54:17, and received the Master of Science degree. He obtained his most significant instruction in the ways of science during this period from James N. Cummins and John Sanford.

In 1985, Kim Dean Bowman began two years of service as a Peace Corps volunteer in the country of Saint Lucia. He was employed as a crop extension specialist, lecturer, and coordinator of agricultural services for the Ministry of Agriculture in that beautiful country. While in Saint Lucia, he met and married the woman of his dreams, Concessa Jean Marie.

Kim Dean Bowman entered the PhD program in horticultural science at University of Florida in 1987. His principal advisor during this period

was Frederick G. Gmitter, Jr., the plant breeder at the Citrus Research and Education Center in Lake Alfred. He received the PhD degree from University of Florida in 1990 and subsequently accepted the post of senior museum scientist with the Citrus Clonal Protection Program and University of California, Riverside.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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Associate Professor
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This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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